Effect of Transthyretin in the brain vasculature
Implications in physiology and pathology

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A todas as pessoas que de alguma
forma ajudaram a tornar-me
no cientista que hoje sou.

"If you can't be a pine on the top of the hill
Be a scrub in the valley-but be
The best little scrub by the side of the rill;
Be a bush if you can't be a tree.

If you can't be a bush be a bit of the grass,
And some highway some happier make;
(…)
If you can't be a highway then just be a trail,
If you can't be the sun be a star;
It isn't by size that you win or you fail-
Be the best of whatever you are!"

[Be the best of whatever you are, Douglas Malloch]

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Abstract

The blood-brain barrier (BBB) is an important barrier that separates and protects the brain from the peripheral circulation. This BBB is formed by special tight junctions, a feature of the brain endothelium that is responsible for the BBB integrity and its low permeability. In several diseases, including Alzheimer's disease (AD), BBB is impaired and changes in some important proteins are found. AD, the most common type of dementia, is characterized by an abnormal extracellular deposition of amyloid-β peptide (Aβ), which is believed to be a consequence of a failure in the balance between its production and clearance. Several molecules have been proposed as Aβ carriers and among them transthyretin (TTR) has been shown to have the capacity to bind and cleave Aβ, preventing its deposition and promoting its clearance, being considered as a neuroprotective protein. Moreover, previous reports highlighted the importance of TTR in the regulation of the expression of central nervous system (CNS) genes, pro-angiogenic genes in the eye and in the umbilical cord and blood-brain barrier genes.

In this project, we aimed at further investigating the participation of TTR in the brain vasculature, in biologic as in pathologic environments.

In order to achieve our objectives, we began by evaluating the effect of TTR in the expression of tight junctions (TJs) - related genes, at the transcript and protein levels, using hCMEC/D3 cells. Our initial results obtained by qRT-PCR, using a PCR array kit, suggested that TTR was modifying the expression of several genes, but further individual qRT-PCR for the most affected genes did not confirm the PCR array results. Moreover, three other genes not evaluated in the PCR array, were assayed by qRT-PCR and no effect of TTR was detected. Next, we decided to advance to the analysis of gene expression at the protein level of two important TJs proteins, occludin (OCLN) and Claudin-3 (CLDN3), by immunocytochemistry. Here, we observed that also protein levels of these TJs were not altered by TTR, as seen at the transcript level. Altogether, our results suggest that TTR is not capable to modulate the gene expression of TJs-related genes.

Furthermore, to evaluate the role of TTR in brain vessel angiogenesis, we examined the expression of genes known to be imported in angiogenesis, using two different endothelial cell lines, hCMEC/D3 and bEnd.3 cells. The qRT-PCR results showed that both cell lines presented similar transcript levels in the absence or presence of TTR variants, indicating that TTR presence also does not influence the expression of angiogenesis-related

genes. Additionally, using an in vitro wound healing assay, we investigated if the endothelial cell lines presented a different migration response, an important process of angiogenesis, when incubated in the presence or absence of TTR variants. However, the migratory response of the endothelial cells was similar in both conditions, as no significant differences were detected in the recovery of the wounded area in cells treated with TTR compared to control. These results supported our qRT-PCR results, demonstrating that TTR variants do not have a modulatory role on angiogenesis in the brain.

Finally, we intended to understand if TTR is involved in the regulation of collagen IV levels in brain vessels. Using AD mice with different TTR backgrounds (TTR+/+, TTR+/- and TTR-/-), by immunohistochemistry, we showed that the collagen IV layer was increased in AD/TTR+/- and in AD/TTR-/-, as compared to AD/TTR+/+ animals. To further understand if TTR is directly responsible for this collagen IV alteration, we measured the collagen IV levels in non-transgenic mice with different TTR backgrounds. Here, we observed that collagen IV levels were similar in all animals, indicating that TTR is not directly responsible for the differences observed in the AD mice. Thus, we hypothesized that Aβ was the responsible for the increase of collagen IV levels in AD animals and, since TTR is capable of binding Aβ promoting its clearance, the differences in collagen IV measured in AD animals may result from an indirect action of TTR. In fact, incubation of bEnd.3 cells with Aβ species induced increased collagen IV which was counteracted by TTR, when co-incubated with Aβ. TTR alone did not alter collagen IV in bEnd.3 cells, further corroborating our observations in the non-transgenic animals, and supporting the explanation that in AD mice, Aβ is responsible for the alterations in collagen IV layer and that TTR, indirectly, delays or partially avoids this alteration by sequestering the peptide, avoiding its aggregation and accumulation, and by enhancing its elimination from the brain.

Previous results from our group also indicated that the protective effect of TTR on Aβ can be improved by stabilizing the tetrameric structure of this protein. With this in mind, also in a previous project, iododiflunisal (IDIF), a potent TTR stabilizer, was administrated to AD mice, resulting in cognitive improvement, as well as in decreased Aβ brain levels. To further investigate the relation between collagen IV levels and the protective role of TTR in AD, we now evaluated its levels in IDIF-treated and in control animals. Our results showed

that levels of collagen IV were decreased upon IDIF treatment, strengthening the stability hypothesis.

Altogether, our results support a neuroprotective role for TTR in AD, mainly by acting directly on Aβ and avoiding, at least some of, the downstream events caused by this peptide. We showed that alterations at the vascular level, such as the thickness of the basement membrane, i.e. collagen type IV layer, can be prevented by TTR. Given that the vascular alterations are thought to be part of the initial alterations in AD, it is of the outmost importance to explore therapeutic strategies aiming at increasing TTR levels, known to be decreased in this disorder.

Key concepts: Blood-brain barrier; Tight-Junctions, Angiogenesis, Alzheimer's disease, amyloid-β peptide, Collagen IV, Transthyretin.

Resumo

A barreira hemato-encefálica (BHE) é uma barreira essencial que tem como função separar e proteger o cérebro da circulação periférica. Esta barreira é conseguida através da existência de junções de oclusão, características do endotélio vascular cerebral, e que são responsáveis por manter tanto a integridade como a baixa permeabilidade da BHE. Em várias doenças, incluindo a doença de Alzheimer (DA), a BHE encontra-se comprometida, sendo possível detetar alterações em diversas proteínas importantes para o funcionamento desta barreira. A DA, o tipo de demência mais comum, é caracterizada por uma deposição extracelular anormal do péptido beta-amilóide (βA), pensando-se que esta deposição se deve a uma falha no balanço entre a produção e a remoção do péptido. Várias moléculas capazes de transportar βA têm sido propostas, entre elas a transtirretina (TTR), proteína capaz de ligar e clivar o βA, prevenindo a sua deposição e promovendo a sua remoção, atuando assim como uma proteína neuroprotetora. Para além desta sua capacidade, publicações anteriores sublinharam a importância da TTR na regulação da expressão de genes no sistema nervoso central, genes pró-angiogénicos no olho e no cordão umbilical e também de genes importantes para a BHE.

Neste projeto, o nosso objetivo foi investigar a participação da TTR a nível vascular no cérebro, tanto em condições fisiológicas como patológicas.

Para atingir este objetivo, começámos por avaliar o efeito da TTR na expressão de genes relacionados com as junções de oclusão, a nível da transcrição e tradução, utilizando a linha celular hCMEC/D3. Os nossos resultados obtidos inicialmente por reação em cadeia da polimerase em tempo real (RCP-TR), utilizando um kit contendo um painel de genes, sugeriu que a TTR estaria a alterar os níveis de expressão de diversos genes, mas após se realizar RCP-TR individuais para os genes mais alterados, os resultados do kit não foram confirmados. Além disso, três genes que não tinham sido avaliados no kit foram analisados individualmente, sendo que também não foram detetadas diferenças. De seguida, decidimos avançar para a análise dos níveis proteicos de duas junções de oclusão importantes, a ocludina e a claudina-3. Aqui, observámos que também os níveis proteicos destas duas junções de oclusão não sofreram alterações pela TTR, resultado idêntico ao que foi observado para os níveis de transcrição. Em suma, estes resultados sugerem que a TTR não é capaz de modular a expressão destes genes relacionados com as junções de oclusão.

De seguida, para avaliar o papel da TTR no processo de angiogénese em vasos cerebrais, avaliámos a expressão de genes que promovem a angiogénese, usando duas linhas celulares diferentes, as hCMEC/D3 e as bEnd.3. Os resultados da RCP-TR demonstraram que, em ambas linhas celulares, os níveis de transcrição dos genes pró-angiogénicos mantiveram-se semelhantes na presença e ausência das variantes da TTR, indicando que a presença da TTR não influencia a expressão destes genes envolvidos na angiogénese. De seguida, utilizando o *in vitro healing assay*, investigámos se as linhas celulares apresentavam diferenças na sua capacidade de migração, um passo importante do processo de angiogénese, quando incubadas na presença ou na ausência das variantes da TTR. Os nossos resultados demonstraram que a migração das células foi semelhante em ambas condições, uma vez que não foram detetas diferenças no encerramento do golpe nas diferentes condições. Estes resultados, em conjunto com os obtidos nas RCP-TR, demonstram que as variantes da TTR não têm uma função moduladora do processo de angiogénese a nível cerebral.

Como último objetivo, pretendíamos perceber se a TTR poderia estar envolvida na regulação dos níveis de colagénio IV nos vasos cerebrais. Para isso, usando murganhos DA e com diferentes genótipos para a TTR (TTR+/+, TTR+/- e TTR-/-), demonstrámos por imunohistoquímica, que a camada de colagénio IV estava aumentada nos animais DA/TTR+/- e DA/TTR-/-, quando comparados com os animais DA/TTR+/+. De seguida, para perceber se a TTR estaria envolvida diretamente nestas alterações do nível de colagénio IV, quantificámos os níveis desta proteína em animais não-transgénicos com diferentes genótipos para a TTR. E, aqui, observámos que em ambos os genótipos, os níveis de colagénio IV eram semelhantes, indicando que a TTR não é diretamente responsável pelas diferenças observadas nos animais DA. Tendo em conta este resultado, colocámos a hipótese de que o βA poderia ser o responsável pelo aumento de colagénio IV nos animais DA e, uma vez que a TTR é capaz de ligar-se ao βA promovendo a sua remoção, as diferenças no nível de colagénio IV observadas nos animais DA podiam ser devido a esta sua ação no βA. E, de facto, ao incubar as células bEnd.3 com diferentes espécies de βA, observou-se um aumento de colagénio IV, aumento este contrariado quando βA e TTR foram incubados simultaneamente. Adicionalmente, quando as células foram incubadas só com TTR, os níveis de colagénio IV mantiveram-se inalterados, corroborando os resultados observados com os animais não transgénicos e apoiando a explicação de que nos animais DA, a TTR é

indiretamente responsável pelas alterações do colagénio IV, através da sua capacidade de sequestrar o βA, evitando não só a sua agregação e acumulação, como também por promover a sua eliminação do cérebro.

No nosso grupo, resultados anteriores indicaram que o efeito protetor da TTR no βA pode ser melhorado através da estabilização da sua estrutura tetramérica. Com isto em mente, também num projeto anterior, um potente estabilizador da TTR, o iododiflunisal (IDIF), foi administrado em animais DA, tendo originado melhorias cognitivas e uma diminuição dos níveis de βA no cérebro. Para investigar uma relação entre o colagénio IV e a função protetora da TTR na DA, avaliámos os níveis de colagénio IV em animais controlo e animais tratados com IDIF. Os resultados desta experiência demonstraram que após o tratamento com IDIF, os níveis de colagénio IV diminuíram, fortalecendo a importância da estabilização da TTR.

Em suma, os nossos resultados apoiam o papel neuroprotetor da TTR na DA, principalmente por atuar diretamente no βA, evitando assim as consequências negativas dos seus efeitos. Demonstrámos que alterações a nível vascular, tal como o aumento da espessura da membrana basal, especificamente da camada de colagénio IV, podem ser prevenidas pela TTR. Uma vez que se pensa que as alterações vasculares podem fazer parte das alterações que ocorrem inicialmente na DA, é extremamente importante explorar estratégias terapêuticas que tenham como objetivo aumentar os níveis de TTR, que se encontram diminuídas nesta doença.

Conceitos chave: Barreira hemato-encefálica; Junções de oclusão; Angiogénese; Doença de Alzheimer; péptido beta-amilóide; colagénio IV; transtirretina

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Abbreviations

ABC	ATP-binding cassette tranporters
AD	Alzheimer's disease
AICD	APP intracellular domain
AJ	Adherent junctions
AMT	Adsorptive-mediated transcytosis
ANGPT	Angiopoietin
APOA1	Apolipoprotein A1
APOE	Apolipoprotein E
APP	Amyloid-beta precursor protein
Aβ	Amyloid-beta peptide
BBB	Blood-brain barrier
BCSFB	Blood-cerebrospinal fluid barrier
bEnd.3	Immortalized mouse brain endothelial cell line
bFGF	Basic fibroblast growth factor
BM	Basal membrane
BSA	Bovine serum albumin
CAA	Cerebral amyloid angiopathy
CLDN	Claudin
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTF	C-terminal fragment
DEAE	Diethylaminoethyl
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
DR	Diabetic retinopathy
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid

FAD	Autosomal dominant familial AD
FAP	Familial amyloidotic polyneuropathy
FBS	Fetal bovine serum
GDNF	Glial-derived neurotrophic factor
GLUT	Glucose transporters
hCMEC/D3	Immortalized human cerebral microvascular endothelial cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFIP	Hexafluoro-2-propanol
hRECs	Human retinal microvascular endothelial cells
HUVECs	Human umbilical vein endothelial cells
IDIF	Iododiflunisal
IGF-I	Insulin-like growth factor I
IGF-IR	Insulin-like growth factor I receptor
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JAM	Junctional adhesion molecules
kDA	Kilodalton
LB	Lubia-Bertani
LRP1	Low-density lipoprotein receptor-related protein 1
LRP2	Low-density lipoprotein receptor-related protein 2
NCBI	National Center for Biotechnology Information
NFT	Neurofibrillary tangles
NVU	Neurovascular unit
OCLN	Occludin
O/N	Overnight
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
Pgp	P-glycoprotein
PMSF	Phenylmethylsulfonyl fluoride
PSEN1	Presenilin 1
PSEN2	Presenilin 2

qRT-PCR	Real-time polymerase chain reaction
RAGE	Receptor for advanced glycation end products
RBP	Retinol binding protein
RT	Room temperature
sAPP	Soluble amyloid-beta precursor protein
SD	Standard deviation
SEM	Standard error of the mean
SLC	Solute carrier
sLRP	Soluble low-density lipoprotein receptor-related protein
SP	Senile plaques
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor beta
TGFB2	Transforming growth factor beta 2
TJ	Tight junctions
Tris	Tris(hydroxymethyl)aminomethane
TTR	Transthyretin
T4	Thyroxine
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR1	Vascular endothelial growth factor receptor 1
VEGFR2	Vascular endothelial growth factor receptor 2
WT	Wild-type
ZO	Zonnula occludens

Introduction

Thesis focus

The main goal of this thesis is to explore the neuroprotective role of a specific protein, called transthyretin (TTR) in one of the most important networks of our brain, the brain vasculature, both in physiology and in pathology. The lack of previous studies on this relation, TTR-brain vasculature, was the initial motivation to start this work, and thus, the next section will provide an overview of relevant topics, introducing key concepts, for a better comprehension of the research project.

1. Blood-Brain Barrier

When we talk about the brain, the first thing that comes to our mind is the complexity of this organ, being active in every second. Although it accounts only for ≈ 2% of the body weight, the brain consumes a large amount of energy, requiring around 20% of an individual's resting metabolic rate (Attwell and Laughlin 2001). To ensure that enough oxygen, glucose and other nutrients are adequately delivered to the brain, it is necessary a large blood vessel and microvessel network, being estimated that the total length of capillaries in the human brain is about 600 km, and the capillary surface area available for molecular transport is about 20 m^2 (Begley and Brightman 2003). But this comes with a problem: despite the advantage of having this large network, undesirable compounds that could be toxic to the brain would be in higher concentrations, which is a problem since the brain is the most critical and sensitive organ in our body (Mahringer, Ott et al. 2014). However, our body is meticulously made, existing a special protection in the central nervous system (CNS) microvessels, the so called blood-

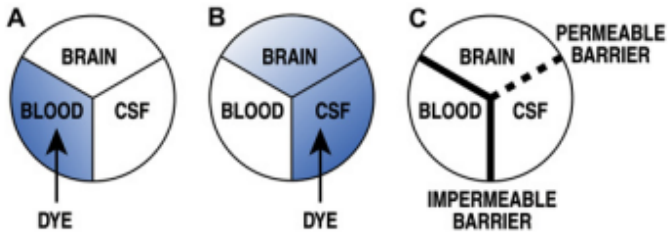

Figure 2. Schematic representation of the diffusion of a dye according to the injection place. (A) and (B) Results of Ehrlich and Goldman experiments, respectively. (C) Assumption of the existence of an impermeable barrier between the CNS and the blood, and a permeable barrier between the brain and the CSF (Zlokovic 2008).

brain barrier (BBB), an exclusive anatomical and physiological barrier separating the CNS and the peripheral circulation. Its existance was firstly hypothezised after Paul Ehrlich has observed that a peripherally injected dye stained peripheral organs but not the brain and the spinal cord (figure 1) (Ehrlich 1885).

Few years later, Goldman performed a staining experiment, similarly to Paul Ehrlich, injecting a dye directly into the cerebrospinal fluid (CSF), after which he observed that the brain and the CSF stained blue, but not the blood (Goldman 1913). These two experiments were essential for the suggestion of the existance of the BBB and, since these discoveries, new information about the BBB has been published for more than 100 years (figure 2).

Year	Discoveries and concepts
1885	Systemic application of a blue dye stained all organs except the brain and the spinal cord [3]
1898	Systemically administered bile acids were not neurotoxic but intracerebrally injected bile acids showed neurotoxicity [5]
1900	Postulation of a barrier between blood circulation and neural tissue to describe the phenomenon [8]
1913	Intrathecal administration of trypan blue results in staining of the brain tissue, whereas intravenous application does not. Definition of the concept of the blood–brain barrier [6]
1921–1922	"Barrière hématoencéphalique" was characterized as a cerebral blood vessel compartment, whereas the choroid plexus epithelium was semipermeable, facilitating the flow of substances from the blood into the CSF [30, 31]
1941	Intracarotid arterial administration of hypertonic solutions caused a transient opening or disruption of the blood–brain barrier explaining the mechanisms behind observed defects at the blood–brain barrier in brain diseases [17]
1942	Friedemann postulated in 1942 that electrochemical properties of injected compounds influence the distribution behavior within the CNS. Thereby, capillaries would be permeable for uncharged and positively charged compounds, but impermeable for negatively charged compounds [18]
1950s	Electron microscopy could not detect an extracellular fluid compartment in the gray matter, which was considered as an explanation for the failure of tracers to enter the brain. Later, this turned out to be an artifact in 1960s
1960s	The presence of extracellular fluid in the cortex was determined by further electron microscopy studies on "freeze-substituted" tissue [20]
1967	Fine structural localization of the blood–brain barrier, demonstration of tight junctions [22]
1969	Visual proof of junctions between endothelial cells [21]
1971	Blood–brain barrier permeability to sugars, amines, amino acids, and neurotransmitters proven by radiolabeled substances [24]
1978	Description of the passage of substances in extracellular fluids from brain to CSF along the CSF "bulk flow" gradient; "sink effect" that removes substances from the brain
1982	Observation of extremely high transendothelial electrical resistances [28]
1980s	Studies in molecular biology of the blood–brain barrier. Cloning and sequencing of glucose transporter gene [29]
1990s	Importance of ABC transporters for barrier function becomes obvious [32]
2000s	Signaling cascades of transporters [33]

Figure 3. History of the blood-brain barrier research and discoveries (Mahringer, Ott et al. 2014).

The BBB is responsible for several roles, as controlling cerebral homeostasis, providing protection against toxic xenobiotics and pathogens (Weiss, Miller et al. 2009), mediating the efflux of waste products and restricting ionic and fluid movements between the blood and the brain (Abbott, Ronnback et al. 2006).

A second "barrier" is formed by the epithelial cells of the choroid plexus, which constitute the blood-cerebrospinal fluid barrier (BCSFB). This one will not be explored, since it was not the focus of our work.

1.1. Neurovascular Unit

The blood-brain barrier exists primarily as a barrier constituted by the cerebral microvascular endothelium, a thin layer of simple cells called endothelial cells (ECs), which are significantly different from those in the periphery (Hawkins and Davis 2005). In close proximity to these brain ECs, other cells are indirectly involved in the development and maintenance of the BBB. All these cells together with the basal lamina, constitute the neurovascular unit (NVU). The first concept of NVU was introduced by Harder (Harder, Zhang et al. 2002), describing a relation between neurons, astrocytes and capillaries. These three types of cells, along with pericytes and basal membrane (BM), create the NVU.

1.1.1. Endothelial cells

Brain ECs are the primary element of the BBB, creating the walls of the capillaries, where a cerebral microvessel is enclosed by a single endothelial cell. These brain ECs are significantly different from non-brain ECs by:

- Absence of fenestrations (Fenstermacher, Gross et al. 1988);
- Presence of intercellular tight junctions (TJs) (Kniesel and Wolburg 2000);
- Enhanced mitochondrial content, associated with a strong metabolic activity (Oldendorf, Cornford et al. 1977);
- Low level of non-specific transcytosis (pinocytotic) (Sedlakova, Shivers et al. 1999);
- Prevented free exchange of solutes between blood and brain (Ohtsuki and Terasaki 2007).

All features together reveal the hallmark of the brain endothelium: a restrict and controlled permeability to plasmatic compounds and ions, protecting the brain from an imbalance in the homeostasis. Endothelial cells are one of the most important components of the NVU, being influenced by the others NVU's cells/BM, as described next.

1.1.2. Basal membrane

Endothelial cells and pericytes are surrounded by the BM, which is constituted by laminin, collagen type IV, proteoglycans, fibronectin and other extracellular matrix proteins produced by both cell types of the NVU (Farkas and Luiten 2001). The BM contribution to the NVU was often underestimated, but it must be considered as an important part of the BBB regulation (Berzin, Zipser et al. 2000). Besides that, disruption of this extracellular matrix is associated with BBB breakdown and increase of its permeability (Rascher, Fischmann et al. 2002), since collagen IV is involved in the regulation of endothelial tight junction protein expression (Savettieri, Di Liegro et al. 2000). Moreover, collagen IV is capable of regulating angiogenesis, which will be discussed in the subchapter 1.4.

1.1.3. Astrocytes

Astrocytes are one of the glial cells that are present in the neurovascular unit. As can be seen in figure 3, these cells communicate with pericytes, neurons and capillary endothelial cells via their several foot processes (Abbott, Ronnback et al. 2006). It has been well documented the importance of astrocytes in the induction and maintenance of BBB integrity (Janzer and Raff 1987, Kuchler-Bopp, Delaunoy et al. 1999). When capillaries are co-cultured with astrocytes, TJs (one of the most important feature in brain endothelial cells) are enhanced in length, width, and complexity, indicating a role in the formation and

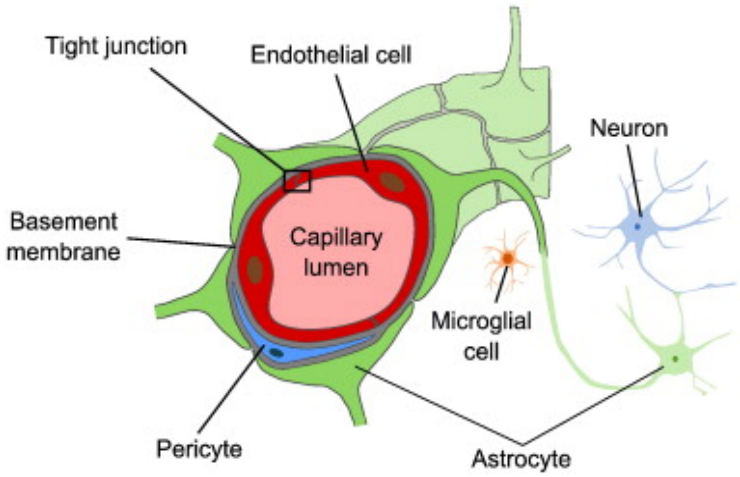

Figure 4. Representation of the neurovascular unit. The BBB is composed by microvessel endothelial cells which are surrounded by pericytes, astrocytes end-feet, neurons and the basal lamina, all essential for the development and maintenance of the BBB (Heye, Culling et al. 2014).

configuration of these TJs (Tao-Cheng, Nagy et al. 1987). Also, astrocytes and endothelial cells are capable to communicate through calcium signals, influencing some aspects of the BBB functioning and transport, such as its permeability (Braet, Paemeleire et al. 2001). Moreover, astrocytes synthesize some biologically active molecules as the transforming growth factor-β (TGF-β), glial-derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF), which may influence endothelial cells (Wilhelm and Krizbai 2014).

1.1.4. Pericytes

Pericytes, also called Rouget cells after their discovery by Charles Rouget, are randomly distributed along the brain and non-brain microvessels, being surrounded (together with ECs) by the basal membrane composed of collagen type IV and other extracellular matrix proteins mentioned above (Bergers and Song 2005). These cells produce substances such as TGF-β, angiopoetin-1 and vascular endothelial growth factor (VEGF), influencing the endothelial function (Wilhelm and Krizbai 2014). Pericytes are important in the formation and maintenance of the BBB since it was shown that pericytes deficiency leads to an increase in the permeability of the BBB (Armulik, Genove et al. 2010), endothelial hyperplasia and abnormal vascular morphogenesis (Hellstrom, Gerhardt et al. 2001). Moreover, the integrity of the wall of the capillaries is compromised by the loss of pericytes (Lindahl, Johansson et al. 1997). It was also shown that pericytes are modulators of blood flow (Peppiatt, Howarth

et al. 2006) and pericytes-derived angiopoietin-1 induces TJs expression, specifically occludin, confirming that pericytes influence positively the maintenance of BBB (Hori, Ohtsuki et al. 2004).

1.2. Junctional complexes at the BBB

As mentioned above, one of the main roles of the BBB is to act as physical barrier, separating the brain from the blood, with a restricted paracellular diffusion and a low permeability across the endothelium. This feature is accomplished by a network of interendothelial junctions as TJs, adherens junctions (AJs) and gap junctions (when present) – figure 4 (Bazzoni and Dejana 2004).

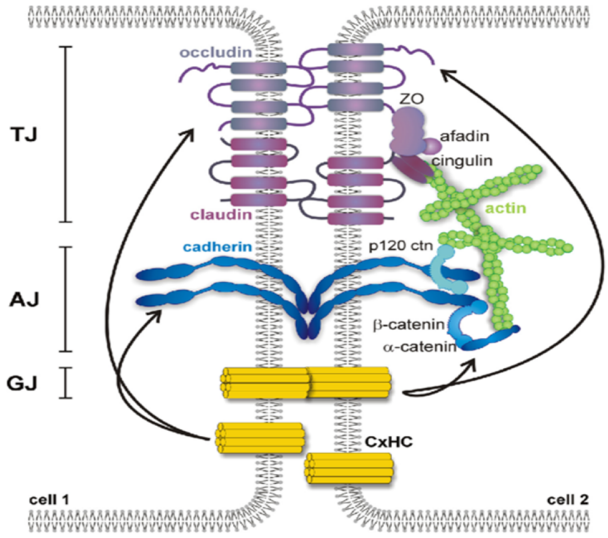

Figure 5. Representation of the interendothelial junctions network. Adjacent endothelial cells are connected by different TJs, AJs and GJs (De Bock, Vandenbroucke et al. 2014).

1.2.1. Tight junctions, adherens junctions and associated proteins

TJs in the brain ECs are very similar to epithelial TJs. In this group we can find a large amount of proteins involved, but it is possible to highlight three major transmembrane proteins (or families): occludin (OCLN), claudins (CLDNs) and junction associated molecules (JAMs). Linked to these proteins we can find different cytoplasmic proteins as

zonula occludens (ZO) family, vinculin and cingulin which are in contact with transmembrane proteins and to the actin cytoskeleton, creating a multi-protein complex (Wolburg and Lippoldt 2002).

The first transmembrane protein identified, firstly in chickens (Furuse, Hirase et al. 1993) and then in mammals (Ando-Akatsuka, Saitou et al. 1996), was OCLN, a 65 kDa protein with four transmembrane domains, whose expression is developmentally regulated. OCLN is highly expressed in the brain endothelium but less (or even absent) in non-neural endothelial cells (Daneman, Zhou et al. 2010). In OCLN deficient mice, TJs are morphologically indistinguishable from the wild-type (wt) controls, indicating that OCLN is not so important for TJs formation (Saitou, Furuse et al. 2000). But, other studies have shown the opposite (Balda, Whitney et al. 1996, Wong and Gumbiner 1997). Still associated with this role, OCLN seems to be an important protein that can alter paracellular permeability (Hirase, Staddon et al. 1997). This protein is mostly associated with the TJ formation but its functions are not limited to this role. As example, OCLN can be involved in epithelial differentiation (Schulzke, Gitter et al. 2005).

With molecular weighs between 20-34 kDa, the CLDN family is constituted by more than 22 members that can be found in different tissues, but only a few are expressed at the BBB (Mahringer, Ott et al. 2014). These are important for the formation and maintenance of the BBB, and their breakdown lead to the disruption of the BBB. In CLDN-5 deficient mice ($CLDN5^{-/-}$ mice), molecules with <800 Da were capable to cross the BBB, even if the development and morphology of blood vessels were not compromised (Nitta, Hata et al. 2003). Moreover, in the same study, CLDN5 downregulation was shown to be related with BBB breakdown via VEGF, although reversible when recombinant CLDN5 was expressed, leading to the rescue of low paracellular permeability and thus, decreasing the BBB breakdown (Argaw, Gurfein et al. 2009).

The other relevant TJs involved in the BBB integrity are the JAM family. There are three known JAMs: JAM-A, JAM-B and JAM-C (or JAM-1, JAM-2 and JAM-3, respectively). These proteins are present in endothelial and epithelial cells, leukocytes, platelets and erythrocytes (Mandell and Parkos 2005). JAM proteins are involved in different

processes, including the regulation of tight junction assembling (Liu, Nusrat et al. 2000) and angiogenesis signaling (Naik, Mousa et al. 2003).

As a part of the junctional complex, it is possible to find other proteins at cell-cell junctions, forming adhesive contacts between cells, the so called adherens junctions (also known as zonula adherens). The AJs complex are mainly composed by the interaction of the cadherin protein family, such E-cadherin and vascular endothelial (VE)-cadherin, and the catenin family including β-catenin, α-catenin and p-120-catenin, which interact with actin (Hartsock and Nelson 2008). Similar to TJs, these proteins are important for the BBB integrity and to reduce its permeability (Corada, Mariotti et al. 1999, Navaratna, McGuire et al. 2007). VE-cadherin, besides its role as a tight connection between cells, is also associated with processes like angiogenesis (Wallez, Vilgrain et al. 2006) and cell proliferation (Caveda, Martin-Padura et al. 1996).

Equally important, transmembrane proteins are associated with cytosolic proteins, some already mentioned above: ZO family, cingulin, vinculin. ZO-1, probably one of the most studied proteins, was shown to be involved in cell-cell tension, angiogenesis, barrier formation (Tornavaca, Chia et al. 2015), and its dissociation is followed by an increase of permeability (Abbruscato, Lopez et al. 2002). Actin is also an important component of this junctional complex. It permits the anchorage of transmembrane proteins (using intermediated proteins) to the actin cytoskeleton, essential for the barrier stability/permeability and cell movement (Lai and Kuo 2005, Dejana, Tournier-Lasserve et al. 2009).

Altogether, we can say that a complex network of proteins is vital for the formation and maintenance of BBB, with innumerous functions, and the failure of one part might be enough to compromise this important barrier.

1.3. Transport at BBB

The transport across the BBB is very controlled, since the maintenance of the homeostasis is crucial for the brain, which is achieved through numerous mechanisms, depending on the nature of the substance crossing the barrier (figure 5). These include

transmembrane diffusion, saturable transporters, receptor-mediated transcytosis and adsortive transcytosis (Banks 2009).

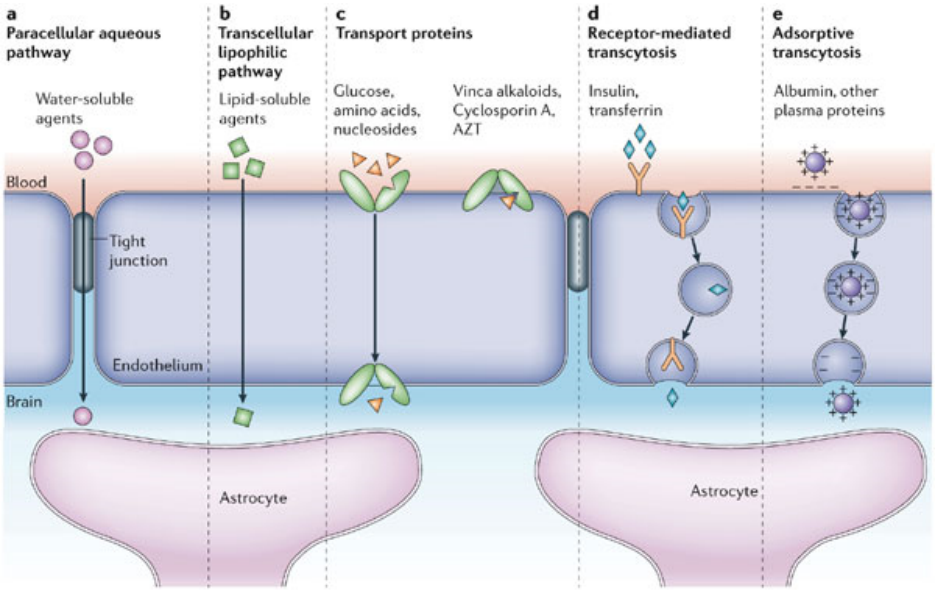

Figure 6. Scheme of transport mechanisms across the BBB, depending on the compound nature (Abbott, Ronnback et al. 2006).

Due to its tight barrier properties, only a few small polar compounds are able to cross the BBB via diffusion across TJs (paracellular transport), including urea and water, since almost no space exists between adjacent cells.

The main type of transport is the transmembrane diffusion (or passive diffusion) (Oldendorf 1974), where molecules can enter inside endothelial cells via a non-saturable mechanism, simply depending on the capacity of the compound to melt into the cell membrane. Small and lipophilic molecules can easily cross the cell membrane, but other factors as charge and tertiary structure also affect this type of transport (Banks 2009). Despite the ability of these molecules to cross the luminal cell membrane, not all will reach the brain. In fact, to regulate passive transport into the brain, endothelial cells have efflux pumps that are responsible to return many unwanted molecules back to the blood (Wong, Ye et al. 2013). This return is made by the ATP-binding cassette tranporters (ABC). At the BBB, the most important ABC transporters for efflux transport are the P-glycoprotein (Pgp), the multidrug resistance-associated proteins and breast cancer resistance protein (Abbott, Patabendige et al. 2010) which remove potentially neurotoxic endogenous or xenobiotic molecules from the

brain, protecting and detoxifying this organ (Dallas, Miller et al. 2006). Oxygen and carbon dioxide are examples of molecules using passive diffusion as the way of crossing the BBB.

Another way for compounds to reach the brain is via carrier-mediated transport. Essential compounds, such as glucose, aminoacids and nucleosides, can enter in the brain using specific transporters. The most common transporter belong to the solute carrier proteins (SLC) family. SLC2A1, more known as glucose transporter-1 (GLUT1), is responsible for the facilitation of glucose transport, following its concentration gradient (Hediger, Romero et al. 2004). Other transporters from the SLC family are involved, such as the monocarboxylate transporters, which transport short-chain monocarboxylic acids (as lactate and pyruvate), and the SLC7 which transport cationic aminoacids (arginine, lysin and ornithine) (Mahringer, Ott et al. 2014). These transporters are crucial for the brain activity since it requires a lot of energy to work, thus, it also needs these metabolic substrates (specially glucose). Ions transporters, such as sodium pump and sodium-hydrogen exchanger, are other type of transporters that are essential for the maintenance and regulation of intracellular pH in the endothelium (Taylor, Nicola et al. 2006).

Proteins and peptides are solutes with a large size and mass and cannot cross the endothelium by passive transport. These molecules reach the brain via vesicular transport across BBB – transcytosis. Two ways of transcytosis can occur: either by specific receptor-mediated transcytosis or nonspecific adsorptive-mediated transcytosis (AMT). The first one is made by specific interaction between the molecules and receptors localized on ECs membrane. Proteins as insulin, leptin and transferrin are examples of proteins transported using this way (Pardridge 2003, Herve, Ghinea et al. 2008). In the AMT, positively charged peptides in the blood interact with the negatively charged phospholipid head groups of the cell membrane, inducing membrane invagination and vesicle formation. After entering the intracellular space, vesicles fuse with the abluminal side of endothelium and are released to the brain. Briefly, it consists in an elctrostatic interaction between molecules and cell membrane (Jones and Polt 2015).

1.4. Development of new vessels

The development of blood vessels can be made via two different processes: angiogenesis and vasculogenesis. In the last one, blood vessels have as origin the differentiation of hemangiogenic stem cells and angioblasts from pluripotent mesenchymal cells (Demir, Kayisli et al. 2006). Angiogenesis, the formation of vessel branches from pre-existing vessels, is the predominantly process of blood vessel formation in the brain (Lee, Han et al. 2009). This process can be subdived in sprouting angiogenesis and intussusceptive angiogenesis (Burri and Tarek 1990). Sprouting angiogenesis is the most studied type of angiogenesis and is composed by different steps: enzymatic degradation of capillary basement membrane, EC proliferation and migration followed by their arrangement in a tube formation, and vascular stabilization through the establishment of new basement membrane and specialized cells attachment (figure 6) (Risau 1997, Adair and Montani 2010).

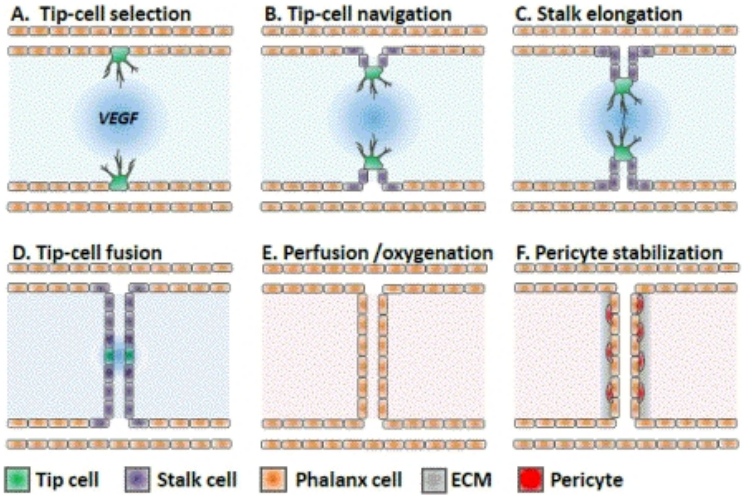

Figure 7. Overview of sprouting angiogenesis (Adair and Montani 2010).

In more detail, the beginning of blood vessels formation starts with the degradation of the BM by enzymes secreted by activated ECs, leading to the creation of little holes in the BM. At this point, these ECs begin to proliferate at a higher rate, and they migrate out through the holes in the BM (Conway, Collen et al. 2001). During that, the tissue in front and around the sprouting vessel is remolded by metalloproteinases, allowing the vessel to extend and to form a tubular structure. To finalize, if the new blood vessel contacts with another existing vessel, it will be stabilized by surrounding of a new BM and the presence of specialized cells,

such as smooth muscle cells and pericytes. Both together provide structural support for the vessel functioning (Zakrzewicz, Secomb et al. 2002, Pandya, Dhalla et al. 2006).

Angiogenesis is highly regulated by the presence of anti-angiogenic and pro-angiogenic factors, allowing to maintain an important balance (figure 7). But, in response to

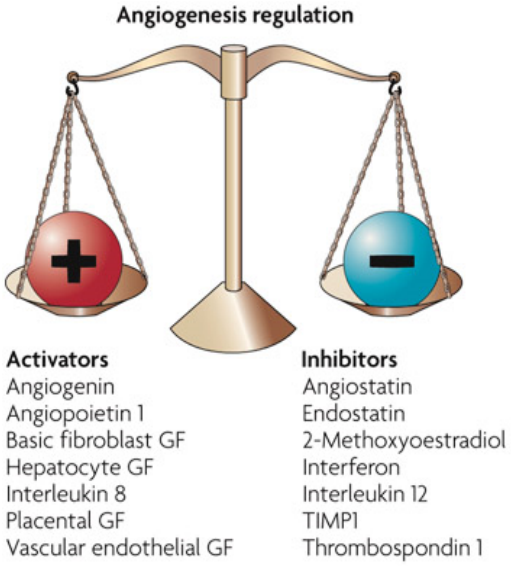

Figure 8. Angiogenesis regulation. Balance between activation and inhibition of angiogenesis is highly regulated by several stimulatory and inhibitory factors in response of several physiological and pathological conditions (Zetter 2008)

several signals, this balance can be altered, both in physiological and pathological conditions.

Physiologically, alterations in the microvessel network are necessary in different processes, including wound healing, exercise and tissue growth (Pries and Secomb 2014). In pathological conditions, angiogenesis can be found up-regulated or down-regulated, depending on the proportion of anti- and pro-angiogenic factors. Up-regulation of angiogenesis can be found in several diseases such as cancer, atherosclerosis and diabetic retinopathy (Folkman 1995), while down-regulation is an important causal factor for coronary artery disease, cardiac failure and tissue failure (Pandya, Dhalla et al. 2006).

Hypoxia, the deprivation of adequate oxygen supply, is an important promoter of angiogenesis. This deficiency of oxygen leads to an increase of pro-angiogenic factors (Hirota and Semenza 2006). bFGF, VEGF and its receptors, angiopoietins (ANGPTs) and transforming growth factor beta 2 (TGFB2) are examples of these pro-angiogenic factors

(Shibuya 2011, Dulloo, Phang et al. 2015). It is important to refer that both type of angiogenic factors are able to affect one or more angiogenic steps, but mainly acting on EC proliferation/migration and on the BM and extracellular matrix (ECM).

1.4.1. Extracellular Matrix functions during Angiogenesis

The ECM is a key component for each step of angiogenesis, providing structural support and also molecular signals that are essential for blood vessel formation. To start angiogenesis, it is required EC activation and proliferation, which is achieved due the action of angiogenic cytokines and EC adhesion to ECM through integrins, since for an efficient cytokine activation, is necessary the presence of integrins (Giancotti and Ruoslahti 1999). Thus, without the presence of ECM, EC activation and proliferation ceases and angiogenesis doesn't occur. Moreover, also EC migration requires adhesion to ECM (Ausprunk and Folkman 1977). Relatively to new blood vessels morphogenesis, ECM are capable to regulate EC shape and morphogenesis. Studies showed that collagen I is capable to alter ECs morphology and to make them align into cords similarly to those observed during angiogenesis in vivo (Whelan and Senger 2003), and also capable of induce and support lumen formation by ECs (Senger and Davis 2011).

1.4.1.1 Collagen IV as a player in angiogenesis

As seen above, the ECM and the BM are important for angiogenesis process. Collagen IV, the predominant protein in the BM, can have different functions in this process. Studies have shown that synthesis and deposition of Collagen IV is indispensable for vascular survival and maturation. Bonanno and colleagues studied the angiogenic response of native ECs in three-dimensional vascular organ culture, culturing rings of rat aorta in the absence or presence of collagen IV, and reported that collagen IV increased the vascular elongation, survival and stabilization, in a dose-dependent manner (Bonanno, Iurlaro et al. 2000). In another study, inhibition of collagen production led to a marked anti-angiogenic effect (Nicosia, Belser et al. 1991). In Bahramsoltani and colleagues work, they showed the expression of collagen IV in four different microvascular endothelial cell types, two of them classified as angiogenic and the other two as non-angiogenic. Curiously, secretion and

deposition of collagen IV was only observed in the two angiogenic cultures, suggesting that angiogenesis is dependent on collagen IV deposition (Bahramsoltani, Slosarek et al. 2014). However, during the ECM remodeling, collagen IV is degraded, generating small anti-angiogenesis molecules from its α chains, such as arresten, canstatin and tumstatin (Mundel and Kalluri 2007). These molecules are endogenous angiogenesis inhibitors which are involved in the balance between pro- and anti-angiogenic factors (Mundel and Kalluri 2007).

2. Alzheimer's Disease

Alzheimer's disease (AD), name given in tribute to the first person that describe it, is a chronic neurodegenerative disease, comprising the major cases of dementia worldwide, that usually affects the older people (age >65 years). The greatest risk factor for AD is age and is characterized by a progressive loss of cognitive functions (such as memory and language) (Burns and Iliffe 2009). The World Alzheimer Report 2016 estimated that there were 46.8 million people worldwide with dementia and this number can reach to 131.5 million in 2050 (International, 2016). Despite of being intensively studied, the etiological mechanisms underlying the neuropathological changes remain unclear, but being suggested that is probably caused by a set of genetic and environmental factors (Reitz and Mayeux 2014).

2.1. Genetics behind Alzheimer's disease

The majority of patients only develop clinical symptoms at age older than 65 years – called late-onset AD or sporadic AD – , but for 2 to 10% of patients the symptoms appear earlier – early-onset AD or autosomal dominant familial AD (FAD) (Van Cauwenberghe, Van Broeckhoven et al. 2016). During a long time, genetic factors contributing to the disease were searched, being discovered several mutations that are capable of triggering the disease. It was found that three important genes are involved as a cause of autosomal dominant familial AD when mutated (originating early-onset AD cases), namely amyloid precursor protein (APP), Presenilin 1 (PSEN1) and Presenilin 2 (PSEN2) (Bertram, Lill et al. 2010), all three genes encoding proteins linked to APP. PSEN1 and PSEN2, members of the same family, are essential components of the γ-secretase complex responsible for cleavage and release of amyloid-β peptide (Aβ). Thus, mutations in these genes interfere with APP

processing and Aβ production (described below). Opposite to FAD, sporadic AD does not show autosomal-dominant inheritance. Instead, multiple genetic and environment risk factors may be involved. One of the genes associated with sporadic AD is the ε4 allele of the apolipoprotein E (APOE) gene, localized on chromossome 19q13. APOE gene can have three different polymorphic alleles - ε2, ε3 and ε4 – which have a worldwide frequency of 8.4%, 77.9% and 13,7% respectively (Liu, Liu et al. 2013), but in AD patients, the frequency of the ε4 allele is about 40% (Bu 2009). Thus, patients who carry APOE ε4 allele are at higher risk to develop AD when compared with noncarriers, but this allele per si is not a determinant of the disease (Schmidt, Carlo et al. 2014). Interestingly, on the other hand, it was shown that ε2 allele could have a protective effect in late-onset AD (Corder, Saunders et al. 1994).

2.2. Pathophysiology of Alzheimer's disease

2.2.1. Neuropathology

In the description of the first case of AD, some typical features were found, both macroscopic and microscopic features. Macroscopically, in the brains obtained from patients with AD, it is possible to identify a clear cerebral cortical atrophy, specially in the primary motor, sensory and visual areas. Also, a symmetrical dilation of the lateral ventricles (hydrocephalus ex vacuo) is observed, caused by a encephalic volume loss (Perl 2010). Although these changes are associated with AD, they may also be present in the brains of elder persons without AD, the difference being the extent and distribution of the changes, which are increased in AD patients (Terry 1986). Microscopically, AD is characterized by the presence of extracellular aggregated Aβ peptide constituting the senile plaques (SP), and intracellular aggregates of hyperphosphorylated tau protein creating the neurofibrillary tangles (NFTs) (figure 8) (Tiraboschi, Hansen et al. 2004, Kolarova, Garcia-Sierra et al. 2012).

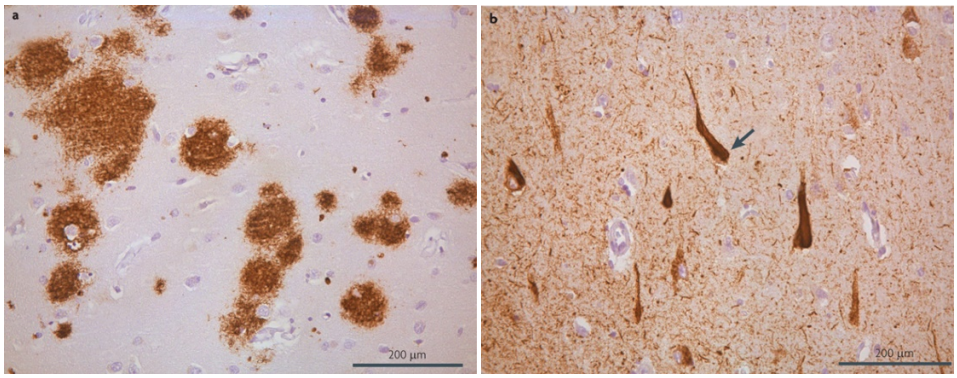

Figure 9. Representation of microscopic hallmarks of AD. (a) Senile plaques and (b) neurofibrillary tangles (Aguzzi and O'Connor 2010).

2.2.1.1. Neurofibrillary tangles

When Alois Alzheimer described the first case of AD, he noted the presence of some abnormal fibrils inclusions inside cells, now known to be the neurofibrillary tangles. The NFTs are composed of abnormal fibrils with around 10nm in diameter that occur in pairs and are twisted in a helical fashion with a regular periodicity of 80nm (Perl 2010). These NFTs are mainly constituted by the microtubule-associated protein tau which presents an abnormal hyperphosphorylation (Lee, Balin et al. 1991), but it is possible to find other proteins, such as ubiquitin (Perry, Friedman et al. 1987) and cholinesterases (Mesulam and Asuncion Moran 1987). Relatively to NFTs distribution, it is possible to find them in the layer II neurons of the entorhinal cortex, the CA1 and subicular regions of the hippocampus, the amygdala, and the deeper layers (layers III, V, and superficial VI) of the neocortex (Morrison and Hof 1997). Tau is a cytosolic protein whose physiologic function is the promotion of the assembly of tubulin into microtubules and its stabilization (Weingarten, Lockwood et al. 1975). Moreover, tau is involved in more than 20 clinicopathological entities, besides AD (Williams 2006). The process which leads to hyperphosphorylation of tau is unclear, but studies suggest to be a consequence of an upregulation of tau kinase or a downregulation of tau phosphatase. Furthermore, these abnormal tau phosphorylations modulate tau aggregation by disrupting its binding to microtubules and leading to its aggregation, resulting in the increase of soluble tau. Next, soluble tau dimerize and forms oligomers. In the end, oligomers originate protomers which will form paired helical filaments and NFTs (figure 9) (Martin, Latypova et al. 2011).

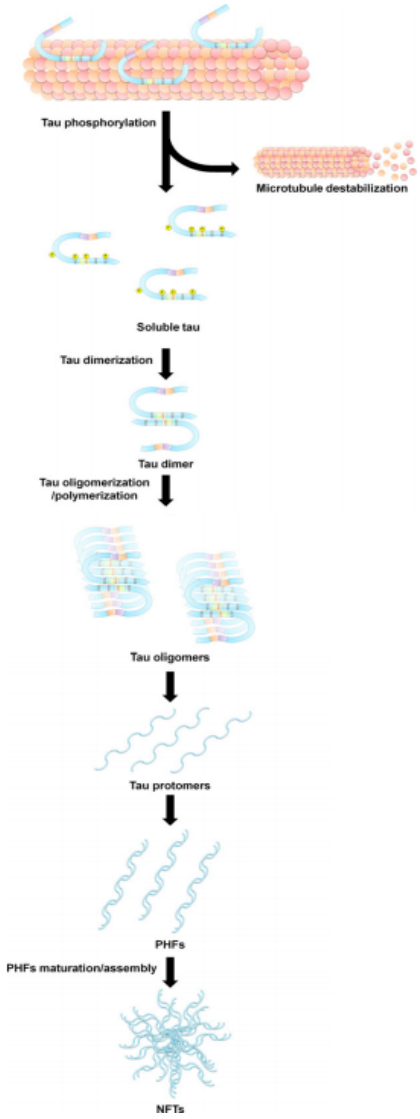

Figure 10. Representation of NFTs formation
(Martin, Latypova et al. 2011).

2.2.1.2. Senile Plaques

Senile plaques, also called neuritic plaques, are extracellular amyloid deposits found in the brain of AD patients, but it is also possible to find in normal aging, being mostly constituted by Aβ (Cras, Kawai et al. 1991). These plaques present a variable morphology and size, and are located specifically in the hippocampus and the cortex (Costa, Ferreira-da-Silva et al. 2008). Also, it is possible to divide amyloid plaques in different subtypes

depending on the morphology, including diffuse SP (pre-amyloid), primitive SP (neuritic), classic SP (dense-core) and compact SP (burnt-out) (Armstrong 2009). The last subtype is characterized by an increase of neurite curvature and dytrophic neurites (axons and dendrites), synaptic loss, neuron loss, and recruitment and activation of both astrocytes and microglial cells (Serrano-Pozo, Frosch et al. 2011).

2.3. Biochemistry of Aβ

As referred above, Aβ is the major component of senile plaques in AD. With approximately 4 kDa, Aβ is a small peptide originated by proteolytic processing of APP, a type I transmembrane protein essential for normal brain (Shariati and De Strooper 2013), where the region that corresponds to the Aβ includes the exon 16 and 17 of APP. Aβ was first isolated and sequenced by Glenner and Wong (Glenner and Wong 1984), and is found in the plasma, brain and CSF. Despite being always associated with its negative effect in AD, Aβ has been linked with several physiological functions such as ion channel modulation (Plant, Webster et al. 2006), regulation of cholesterol transport (Yao and Papadopoulos 2002) and more recently suggested as an antimicrobial peptide (Kumar, Choi et al. 2016).

APP is a protein abundantly expressed in the brain but is possible to find it in other tissues. The gene codifying for APP is located in the chromosome 21 in humans, and contains 18 exons (Zheng and Koo 2011). Interestingly, since APP gene duplication alone is capable to cause early-onset AD with cerebral amyloid angiopathy, it explains the increased risk for Down's syndrome (trisomy 21) patients to develop AD (Thinakaran and Koo 2008). APP processing can occur in two different ways: the non-amyloidogenic pathway and the amyloidogenic pathway, being Aβ produced by the later (figure 10).

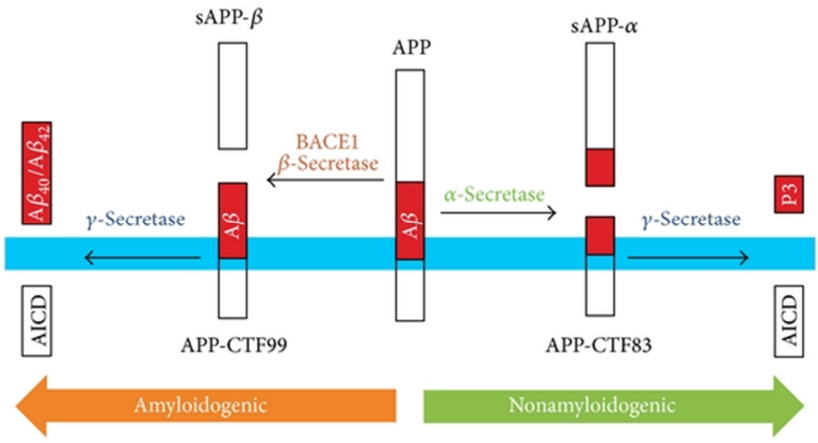

Figure 11. Representation of APP processing. The transmembrane APP can be processed by two different pathways: in the left it is represented the amyloidogenic pathway and in the right the non-amyloidogenic pathway (Pajak, Kania et al. 2016).

2.3.1. APP processing: non-amyloidogenic and amyloidogenic pathways

Depending on the proteolysis pathway, APP can originate different peptides which have different functions. In the non-amyloidogenic pathway, two enzymes are responsible for the cleavage of APP. Firstly, α-secretase, an enzyme of the ADAM family (Rossner 2004), cuts the peptide bond between the Lys687-Leu688 of APP770, within the residues 16 and 17 of Aβ sequence, abolishing its production (Esch, Keim et al. 1990). This cleavage originates a large soluble fragment (sAPPα, ~100kDa) and a carboxyl-terminal fragment (CTF83, ~10kDa). The following cleavage is made by γ-secretase which cuts the CTF83, generating the p3 peptide and an APP intracellular domain (AICD, ~6kDA) fragment (Multhaup, Huber et al. 2015). Importantly, all peptides resulting from both cleavages are non-amyloidogenic. Also relevant, γ-secretase is a complex of four proteins, where the PSEN1 and PSEN2 are involved. Mutations in the genes coding these two proteins lead to an increase of Aβ production (enhances Aβ42 production compared to Aβ40), being one of the explications for the importance of these mutations being a cause of early-onset AD.

In a very similar process, in the amyloidogenic pathway, APP is also cleaved by two enzymes. Here, the difference is in the enzyme responsible for the first cut, the β-secretase instead of the α-secretase, generating the soluble ectodomain sAPPβ and the CTF99 fragment after the cleavage (Zhang, Thompson et al. 2011). The CTF99 fragment is then cleaved by

the γ-secretase, releasing the AICD fragment and the Aβ peptide (Multhaup, Huber et al. 2015). This Aβ peptide can have different sizes and, consequently, it has different propensity to oligomerize and form the amyloid plaques (Haass and Selkoe 2007). But, it needs to be clear that APP processing occurs in the normal cellular metabolism through life, being possibly to find Aβ and NFTs in the brain of non-AD elderly people (Terry 1986). But then, how Aβ is related as a cause of AD?

2.3.2. Amyloid cascade hypothesis

The etiology of AD is still unclear, and different theories try to explain this disorder, all based in different molecular mechanisms to back them up, but not absolutely consistents.

Among these theories, the "amyloid cascade hypothesis" is the most known and stronger. It was formalized by Hardy and Higgins (Hardy and Higgins 1992). The beginning of this cascade is when changes in Aβ metabolism occur, such as increase in total Aβ production, increase in the Aβ42 specie compared to Aβ40, since the first is more toxic, or reduction in Aβ degradation/clearance (Selkoe 1991). Then, Aβ assembly occurs (figure 11), starting with its oligomerization and formation of non-fibrillar deposits. These oligomers are

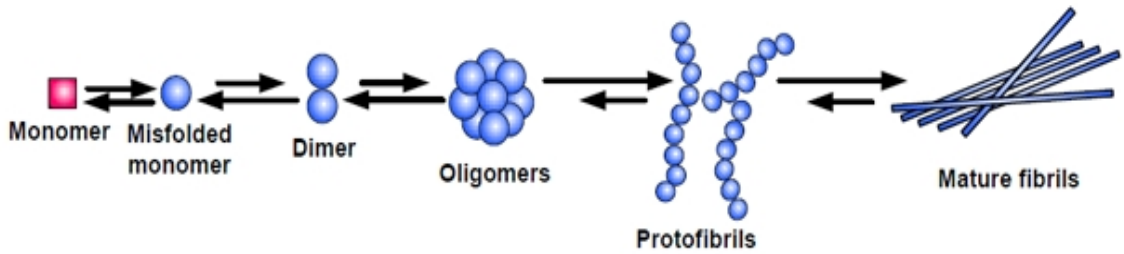

Figure 12. Representation of Aβ assembly. Adapted from (Kumar and Walter 2011).

already capable to do severe changes in synaptic function, leading to neuronal dysfunction. In fact, soluble Aβ oligomers represent the most toxic form of this protein (Kumar and Walter 2011). Over time, the non-fibrillar deposits evolve to Aβ fibrils, and several negative events occur: local inflammatory responses (as microgliosis and astrocytosis), synaptic spine loss, neuritic dystrophy, oxidative stress and altered ionic homeostasis. Finally, oligomerization and hyperphosphorylation of tau is observed. All changes together lead to a widespread neuronal dysfunction, cell dead and culminates in dementia with senile plaques and NFTs

(Haass and Selkoe 2007). Nevertheless, this hypothesis is not accepted by everyone, since the accumulation of new studies show data contradicting this theory. For example, it was shown a poor correlation between the degree of deposition and the degree of dementia in AD patients. It was also observed that some mouse models of AD show behavioral deficits prior to amyloid deposition (Pimplikar 2009).

All together, it is clear that the pathogenesis of AD is a complex process and several factors are involved, and it can not be explained by a single theory.

2.3.3. Aβ clearance

It is known that in the brains of AD patients, Aβ is highly present, but how it happens if the majority of patients with sporadic AD do not present an increase of Aβ production? The unbalance between Aβ production and clearance is determinant for Aβ accumulation. Since dysfunction in its clearance is crucial in this process, it is also important to understand the mechanisms behind it (Wang, Zhou et al. 2006).

The clearance of Aβ from the brain can occur by different pathways: receptor-mediated transport across the BBB, enzyme-mediated degradation and anti-Aβ autoantibodies.

The transport of Aβ across the BBB is possible to happen in both direction: brain-to-blood and blood-to-brain. From the brain to the blood, it can be removed via interstitial fluid bulk flow into the bloodstream (Crossgrove, Li et al. 2005) or be transported across the BBB via receptor mediated, the principal mechanism of Aβ transport across this barrier. Moreover, removal of Aβ via interstitial fluid occurs very slowly and it is responsible for the clearance of only 10-15% of the total brain Aβ (Wang, Zhou et al. 2006).

During receptor-mediate brain efflux across the BBB, Aβ removal starts with its binding to the Low-density lipoprotein receptor-related protein 1 (LRP1) at the abluminal side of the endothelial membrane, crossing the BBB by endocytosis or transcytosis, and reaching the blood. There, the free Aβ circulating in the blood is sequestered by soluble LRP (sLRP) and guided to its degradation organs, the liver and the kidney (systemic clearance) (Bell and Zlokovic 2009). In addition to LRP1, other transporters are involved in efflux of Aβ, such as LRP2 (also called megalin) and P-gp (Jeynes and Provias 2013).

As said before, Aβ can also enter in the brain from the periphery via receptor-mediated transport. Here, the receptor for advanced glycation end products (RAGE) is the major "helper", being located on the luminal membrane of the endothelium.

In conclusion, the balance between influx and efflux of Aβ, mediated by LRP1/2 and P-gp or RAGE, is important to maintain a reasonable Aβ level in the brain. Alterations on the levels of these receptors is enough to lead to an impaired clearance of Aβ and consequently, to all the negative effects expected in the amyloid cascade hypothesis.

2.4. BBB dysfunction and AD

In several diseases, such as AD, BBB dysfunction has been described as an important part in both early and late steps of disease progression (Weiss, Miller et al. 2009). In fact, important features of the BBB described here are negatively altered, contributing for the exacerbation of the AD (figure 12).

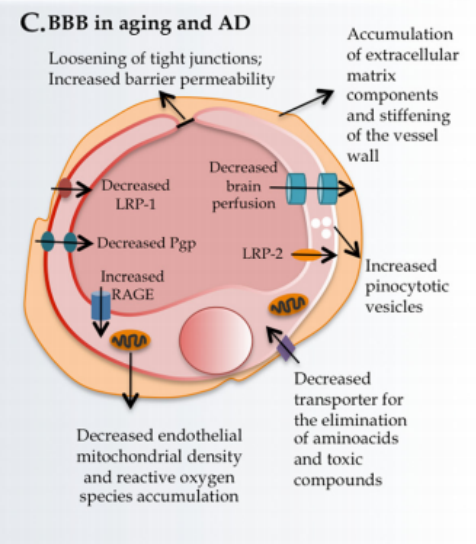

Figure 13. Alterations leading to BBB dysfunction in aging and in AD (Marques, Sousa et al. 2013).

First, numerous studies demonstrated that Aβ is capable to decrease the levels of tight junction proteins and, consequently, increase the permeability of BBB (Tai, Holloway et al. 2010, Kook, Hong et al. 2012).

Several alterations are also observed in the Aβ influx and efflux, mostly due to alterations in the levels of the transporters. LRP1, the major mediator of Aβ efflux, was

demonstrated to be decreased in AD (Shibata, Yamada et al. 2000). Moreover, it was shown that P-gp, the other protein involved in Aβ efflux is lower in AD (Vogelgesang, Cascorbi et al. 2002, Wijesuriya, Bullock et al. 2010). On the other hand, several studies showed that RAGE is found to be upregulated in AD (Srikanth, Maczurek et al. 2011). All together, these alterations contribute to decreased Aβ efflux and increased influx, and consequently to disease progression.

Other alterations can be found, such as accumulation of extracellular matrix components and stiffening of the vessel wall, increased pinocytotic vesicles and decrease of endothelial mitochondrial density (Marques, Sousa et al. 2013).

2.4.1. Cerebral amyloid angiopathy – Aβ type

Cerebral amyloid angiopathy (CAA) is a disorder caused by the accumulation of amyloid in the walls of the brain's blood vessels, being classified according to the amyloid protein involved (Yamada 2015). The most common form of CAA is the accumulation of Aβ (figure 13), and it is commonly found in brains of AD patients and also in elder brains without AD (Vinters 1987), however CAA can be an independent pathogenic factor contributing to

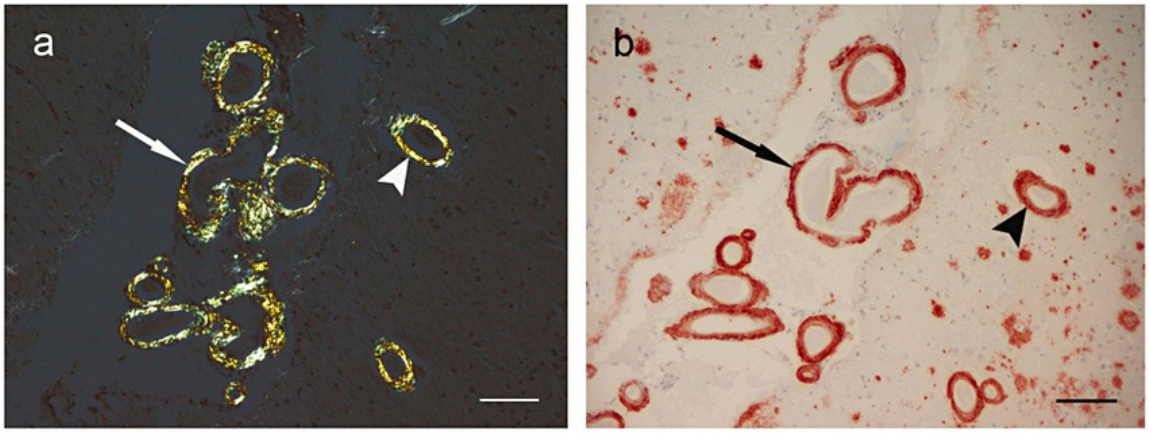

Figure 14. Representation of brain's blood vessels in CAA. Deposits of amyloid are observed (a) using histological Congo red staining, using polarized light and (b) immunohistochemistry against Aβ (Tanskanen, Makela et al. 2012)

dementia. Similar to AD, the prevalence of CAA increases with age, and mutations in the APP gene are associated with this disease. Some studies suggested that Aβ found in vessels is derived from the brain cells, being transported through periarterial interstitial fluid drainage pathways until reach the blood vessels for its clearance (Weller, Massey et al. 1998). Since the mechanism of clearance is compromised, Aβ starts to progressively accumulate.

Aβ accumulation can also result from an increase of the BM in vessels: the thickness of the BM can lead to a formation of a barrier, preventing Aβ to reach the blood. Thus, Aβ slowly accumulates, creating all the negative effects already described. Moreover, it is known that Aβ has affinity to proteoglycans present in the BM (Snow, Kinsella et al. 1995, Weller, Massey et al. 2000). The presence of these proteoglycans in the BM might sequester Aβ from the interstitial fluid, leading also to accumulation of Aβ (Tian, Shi et al. 2006). Alterations in the BM are present in AD and CAA and, in fact, several studies evaluated the levels of collagen IV in AD brains. In most of the studies performed with mice, it was observed an increase in collagen IV in AD brain vessels compared to control littermates (Bourasset, Ouellet et al. 2009, Mehta, Short et al. 2013). Moreover, similar results were obtained when comparing human brains of AD patients with controls (Kalaria and Pax 1995, Lepelletier, Mann et al. 2017). Interstingly, aging is also associated with increased collagen IV content in human microvessels (Uspenskaia, Liebetrau et al. 2004). This last result is specially important, since CAA can occur in elder brains without AD.

3. Transthyretin

3.1. TTR – Structure and functions

TTR, previously called prealbumin due to the ability to migrate slightly faster than albumin on an electrophoresis of a plasma sample, is a protein mainly synthesized in the liver and in the choroid plexus, being secreted into the bloodstream and the CSF, respectively (Soprano, Herbert et al. 1985). The TTR gene is localized in the chromosome 18 (Wallace, Naylor et al. 1985) and codifies for the TTR-monomer originating a polypeptide with 147 aminoacids. TTR is a homotetrameric protein, having four identical subunits assembled, originating a protein with ~55 kDa (Kanda, Goodman et al. 1974). It is possible to find TTR (or a homologous protein) in many species, including mammals, birds, reptiles and amphibians (Schreiber and Richardson 1997, Power, Elias et al. 2000).

The main known physiological role of TTR is the transport of thyroid hormone thyroxine (T4) (Palha 2002) as well as of retinol (vitamin A) that is bound to retinol binding protein (RBP) (Butler, Chan et al. 2016). TTR in blood transports about 15% of plasma T4

whereas TTR in CSF transports approximately 80% of the available T4. This difference is mostly due to the presence of other T4 transporters in blood, such as thyroxine binding protein and albumin (Chen, Chen et al. 2016). Despite its role in transport, TTR knockout mice did not show impaired T4 or retinol metabolism (Palha, Hays et al. 1997, Sousa, de Escobar et al. 2005).

TTR was also described as a protease, capable of cleaving apolipoprotein A1 (ApoA1) *in vitro*, and lipidated ApoA1, a relevant action since this cleavage decreases its ability to promote the efflux of cholesterol (Liz, Faro et al. 2004, Liz, Gomes et al. 2007). Furthermore, another proteolytic action by TTR was found: TTR is capable to cleave Aβ peptide, suggesting that TTR could be involved in Aβ clearance. Moreover, the originated Aβ species present reduced amyloidogenic potential, as compared to the full-length peptide (Costa, Ferreira-da-Silva et al. 2008). Liz et al. also showed that TTR is able to cleave amidated neuropeptide Y. All together these data suggests that TTR has natural substrates in the nervous system (Fleming, Mar et al. 2009, Liz, Fleming et al. 2009).

TTR was also shown to be neuroprotective in the central nervous system, namely in ischemia (Santos, Lambertsen et al. 2010), regeneration (Fleming, Mar et al. 2009) and memory (Sousa, Marques et al. 2007).

3.2. TTR as a cause of disease

TTR is the key protein in Familial Amyloid Polyneuropathy (FAP), a neurodegenerative disease caused by the accumulation of mutant TTR in several organs and tissues, with a special involvement of the peripheral nervous system (Hammarstrom, Wiseman et al. 2003). Other pathologies associated to TTR aggregation are senile systemic amyloidosis and familial amyloid cardiomyopathy (Buxbaum and Reixach 2009).

FAP was firstly described by Dr. Corino de Andrade (Andrade 1952), and is a hereditary autosomal dominant disease. A large number of mutations are already described, being the substitution of a valine residue for a methionine at position 30 (V30M TTR) the most common (Saraiva, Birken et al. 1984, Fleming, Mar et al. 2009). Other mutations have been studied and associated with very different clinical phenotypes/roles. For example, L55P

TTR is associated with one of the most aggressive forms of TTR-related amyloidosis (Jacobson, McFarlin et al. 1992). However, T119M TTR is characterized as a non-aggressive mutation that has a protective role against the disease (Almeida, Alves et al. 2000). It is suggested that the amyloidogenic potential of TTR variants depend on its tetrameric stability and it is the dissociation of the tetramer into monomers the basis of a series of events that will lead to TTR amyloid formation (Cardoso, Goldsbury et al. 2002).

3.3. TTR as a neuroprotective protein in AD

Since Schwarzman and colleagues, the first group to find a relation between TTR and Aβ, suggested that TTR could have a neuroprotective role in AD, several studies have been done to understand the relationship between TTR and Aβ. In their study, Schwarzman and colleagues showed that TTR is the major Aβ sequestering protein in CSF, preventing amyloid formation (Schwarzman and Goldgaber 1996). Moreover, this was highlighted by the observation that AD patients present decreased TTR levels in the CSF (Hansson, Andreasson et al. 2009) and in plasma/serum (Ribeiro, Santana et al. 2012). Importantly, TTR levels correlate with disease state, meaning that TTR could be a biomarker (Ribeiro, Santana et al. 2012, Velayudhan, Killick et al. 2012).

In vivo data obtained from AD transgenic mice established in different TTR genetic backgrounds demonstrated that the genetic reduction of TTR resulted in increased Aβ amyloid burden and total Aβ brain levels (Oliveira et al, 2011).

Furthermore, Costa and colleagues revealed that TTR is capable of binding to different Aβ species: soluble, oligomers and fibrils (Costa, Goncalves et al. 2008), and even of inhibiting and disrupting Aβ fibrils, suggesting this inhibition/disruption as a protective role of TTR in AD. Moreover, in a study performed using different TTR variants, it was shown that T119M presented the highest affinity to Aβ and L55P the lowest, suggesting that amyloidogenic potential of TTR is correlated inversely with binding affinity to Aβ (Costa, Goncalves et al. 2008). In the same work, however, authors showed that TTR L55P has the ability to inhibit Aβ fibril formation and even disrupt fibrils in a similar level as WT TTR, as shown by electron microscopy (Costa, Goncalves et al. 2008). However, the following experiments, using more sensitive assays, showed that L55P does not inhibit Aβ oligomer

formation nor protects against Aβ toxicity (Ribeiro, Saraiva et al. 2012). Very recently, it was suggested that TTR proteolytic activity is required for the neuroprotective effect of the protein (Silva, Eira et al. 2017).

Also recently, Alemi and colleagues showed that TTR participates in Aβ brain efflux at the BBB (Alemi, Gaiteiro et al. 2016). Using the hCMEC/D3 cell line as an in vitro model of the BBB, these authors showed that TTR, added to the brain side of the transwell system, was capable of increasing Aβ transport from the brain to the blood side. However, TTR added to the blood side had no effect the brain-to-blood permeability of hCMEC/D3 to Aβ peptide, indicating that TTR interacts directly with the peptide, probably transporting Aβ to its efflux receptor at the membrane. Interestingly, authors showed that TTR can cross in the brain-to-blood direction but not in the blood-to-brain way, showing that TTR can participate in Aβ removal from the brain but cannot be responsible for its entry back into the brain, re-enforcing a neuroprotective role for TTR in AD.

3.3.1. Importance of TTR stabilization

The idea involving the importance of TTR stabilization appeared in 1993, when McCutchen and colleagues showed the ability of L55P TTR to denaturate to the amyloidogenic intermediate at pHs where WT TTR remained stable, indicating a difference in the stability between both variants (McCutchen, Colon et al. 1993).

To explore the state of TTR in AD, the binding between TTR and T4 was assessed using plasma from AD patients, showing a decreased ability of TTR to carry the hormone, indicating that this function was compromised (Ribeiro, Santana et al. 2012). This could suggest that TTR is unstable in AD, leading to its faster clearance, explaining its lower levels. These authors hypothesized that TTR stability is a key factor in the TTR-Aβ interaction and, consequently, that TTR tetrameric stabilization by small chemical compounds such as iododiflunisal (IDIF) and resveratrol would improve TTR binding to Aβ and AD features.

In fact, IDIF administered orally to an AD mouse model resulted in decreased brain Aβ deposition, decreased Aβ levels in plasma, and also in improvement in cognitive functions associated to AD (Ribeiro, Oliveira et al. 2014). Moreover, in another study, resveratrol was administered in the diet of AD mice during two months, resulting in an

increase of TTR plasma levels and a decrease of brain Aβ plaque burden (Santos, Rodrigues et al. 2016). Very recently, Alemi and colleagues showed that TTR stability is critical in assisting Aβ clearance (Alemi, Silva et al. 2017). Overall, these results demonstrate that stabilization of TTR could be a promisor therapeutic strategy in AD.

3.4. TTR as a CNS gene modulator

The BBB is an important barrier in physiology and pathology, and because of that, several groups have been studying the influence of molecules in BBB features, being TTR one of them.

Recently, studies have been done to unravel if TTR is a modulator of BBB, analyzing possible alterations in angiogenesis and gene expression. In fact, besides the direct action of TTR in Aβ, TTR can alter positively some important features of BBB.

Initially, TTR was described as a molecule involved in regulation of Insulin-like growth factor receptor I (IGF-IR), having the capacity to increase IGF-IR levels in the hippocampus, both at transcript and protein level, demonstrated by studies in WT vs TTR-/- mice and in vitro-cultured cells (Vieira, Gomes et al. 2015). Later, the same group showed a synergy action of TTR and IGF-I in one of the IGF-IR signaling pathways, the Akt pathway. Here, TTR alone could not activate Akt pathway, but when it was simultaneously added with IGF-I, originated a stronger response compared with IGF-I alone (Vieira, Leal et al. 2016).

Work by Alemi and colleagues showed that the ability of TTR to enhance Aβ brain efflux can result not only from a direct TTR/Aβ interaction and transport across the BBB, but also from capacity of TTR to modulate positively LRP1 levels (Alemi, Gaiteiro et al. 2016), authors showed that both LRP1 transcript and protein levels were increased in TTR+/+ mice brains compared to TTR-/-; this result was confirmed in hCMEC/D3 cells that showed increased LRP1 levels in the presence of recombinant human with TTR. Altogether, authors propose that TTR is capable of mediating Aβ clearance through LRP1 (both at the brain and liver) (figure 14) (Alemi, Gaiteiro et al. 2016).

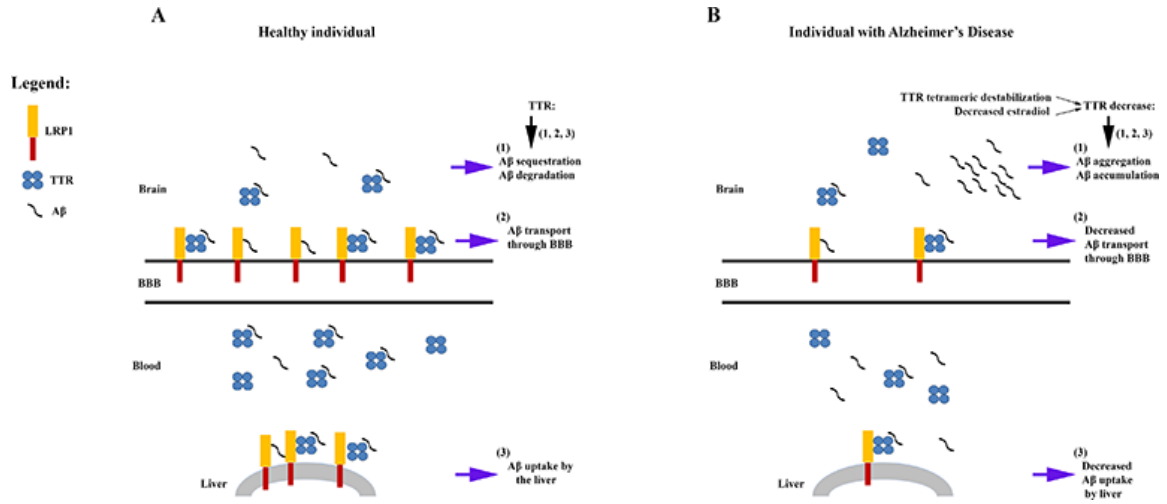

Figure 15. Representation of TTR-mediation in Aβ clearance (Cardoso I et al 2016)

Relatively to angiogenesis, it was demonstrated the capacity of TTR to regulate angiogenesis, by comparing the expression of several pro-angiogenic genes, such as VEGF1 and VEGFR2, in human umbilical vein endothelial cells (HUVECs), using WT TTR and V30M TTR. V30M TTR was capable to modulate negatively the expression of these genes compared to WT TTR. Moreover, V30M TTR inhibited endothelial cell migration using a wound healing assay (Nunes, de Oliveira et al. 2013). In a similar study, Shao and colleagues, trying to understand the effects of TTR in diabetic retinopathy (DR), compared the levels of pro-angiogenic genes in human retinal microvascular endothelial cells (hRECs) cultured with TTR in natural and simulated DR environments (hyperglycemia and hypoxia). Here, they observed that TTR led to the repression of cells proliferation and the development of neovascularization in a DR environment (Shao and Yao 2016).

In summary, several studies highlight the importance of TTR as an important neuroprotective protein in AD through the sequestering and cleavage of Aβ peptide, and by assisting its elimination across the BBB, promoting its clearance. Moreover, TTR is also suggested to modulate positively the BBB, and thus it is possible that this protein impacts at the vascular system in other ways.

Nevertheless, the ability of TTR to regulate angiogenesis in the brain has never been assessed, either in biological or in pathological conditions. Also, the interplay between TTR and other players in angiogenesis has not been studied. Finally, and although the genetic

reduction of TTR in AD mouse models results in increased Aβ brain deposition, AD features associated to vessel disease have not been evaluated in these models. Thus, this project aims at unravelling the possible relation between TTR and the brain vascular system

Objectives

In this project, we aimed at investigating the impact of TTR in the brain vasculature. Previous reports implicated TTR in the regulation of the expression of pro-angiogenic genes in the eye and in the umbilical cord and thus, we aimed at further investigating the participation of TTR in brain vasculature, in biologic as in pathologic environments.

In order to do that, our experiments had the following goals:

1. To investigate the influence of TTR in the blood-brain barrier:
    a. Ascertain the effect of TTR in the gene expression of TJs-related genes at the transcript level using a global approach, the PCR array, and the traditional approach qRT-PCR, using hCMEC/D3 cells;
    b. Evaluate in hCMEC/D3 cells, the influence of TTR in the gene expression of TJs-related genes at the protein level by immunocytochemistry;
    c. Explore the modulator role of TTR on the angiogenesis capacity of two different types of immortalized endothelial cells, hCMEC/D3 and bEnd.3 cells, by qRT-PCR and in vitro wound healing assay.
2. To assess whether TTR impacts on brain vasculature, specifically in the basal membrane, in AD and non-transgenic mice:
    a. Evaluate and compare the levels of collagen IV in brain vessels of AD/TTR$^{+/+}$, AD/TTR$^{+/-}$ and AD/TTR$^{-/-}$ female mice, at seven months, by immunohistofluorescence;
    b. Identify how TTR is implicated in collagen IV changes in brain vessels using a non-transgenic model;
    c. Confirm the involvement of TTR in the alteration of collagen IV levels in AD/TTR$^{+/-}$ mice treated or non-treated with IDIF;

d. Explore the role of Aβ in the thickening of the BM, namely in the collagen IV layer, in cell culture, using the bEnd.3 cell line.

Material and Methods

1. Recombinant TTR production and purification

Human recombinant TTR variants were produced in a bacterial expression system using Escherichia coli BL21 (Furuya, Saraiva et al. 1991) and purified in a similar way as described before (Almeida, Damas et al. 1997).

Firstly, E. coli BL21 were transformed with pET plasmids carrying TTR variants cDNA (WT TTR, V30M TTR or L55P TTR). Transformed bacteria were grown overnight (O/N) in 15-ml starter cultures of Lubia-Bertani (LB) medium containing 50 μg.mL^{-1} of ampicillin at 37ºC and 180 rpm. One-liter cultures with LB containing ampicillin were inoculated with starter culture and incubated at 37ºC and 180 rpm until reach an optical density of 0.4-0.5. Then, TTR expression was induced with 500 μM of Isopropyl β-D-1-thiogalactopyranoside (IPTG), and the culture was grown for 6 h at 37ºC and 180 rpm. Next, culture media was centrifuged at 4000 rpm for 10 min at 4ºC and the supernatant was discarded. The pellet was resuspended in lysis buffer (1M Tris pH 7.5, 0.5 M Ethylenediamine tetraacetic acid (EDTA), Triton-X, Phenylmethylsulfonyl fluoride (PMSF) and 10 μg.mL-1 DNAse), and bacterial cell disruption was performed by successively freeze-thaw-freeze the solution and by sonication. In order to remove cell debris and obtain only the soluble proteins, it was performed a centrifugation at 15000 rpm for 25 min at 4ºC. Pellet was discarded and the supernatant was dialyzed O/N in glycine-acetate at 4ºC.

For TTR purification, firstly, we performed an ion-exchange chromatography. The dialyzed solution was passed through an ion exchange column of diethylaminoethyl (DEAE)-cellulose (Sigma). The column was washed with glycine-acetate buffer, pH=7.0, and the fraction containing TTR was eluted by increasing the ionic strength, using an solution of glycine-acetate +3% sodium chloride. Then, this fraction containing TTR protein was dialyzed against water and lyophilized. TTR was finally isolated by preparative electrophoresis in a native Prosieve agarose (Lonza) gel. The slice of the gel containing TTR was cut and eluted in 38 mM glycine and 5 mM Tris. TTR variants purity was analyzed by Coomassie blue staining and protein identification by Western Blot and by Peptide Mass Fingerprint (PMF+MALDI-TOF). Protein concentration was determined using the Bradford Method, using bovine serum albumin (BSA) as standard.

2. Preparation of Aβ species

Synthetic $A\beta_{42}$ (Genscript) was dissolved in hexafluoro-2-propanol (HFIP) (Sigma) and kept at room temperature (RT) overweekend. After, the HFIP was removed under a stream of nitrogen and the powder was dissolved in DMSO at 2 mM. From this stock, three different species of $A\beta_{42}$ were produced: soluble, oligomers and fibrils. $A\beta_{42}$ was diluted to 100 µM in Ham's F12 medium and then incubated at 4ºC during three days for oligomer formation or at 37ºC for seven days for fibril formation. Soluble $A\beta_{42}$ was obtained by diluting the peptide to 100 µM in Ham's F12 medium immediately before adding to cells. To confirm the presence of the different $A\beta_{42}$ species, samples were visualized by transmission electron microscopy.

3. Transmission electron microscopy

For visualization by transmission electron microscopy (TEM), samples aliquots of $A\beta_{42}$ species were absorbed to carbon-coated collodion film supported on 300-mesh zinc grids, and negatively stained with 1% uranyl acetate. The grids were visualized with a JOEL JEM-1400 transmission electron microscope equipped with an Orious Sc1000 digital camera.

4. Cell culture

Two different cell lines were used as a model of BBB in this work: the immortalized human cerebral microvascular endothelial cell line (hCMEC/D3 cell line) , purchased at Tebu-Bio, and the immortalized mouse brain endothelial cell line bEnd.3, which was kindly provided by Dr. Teresa Summavielle (I3S, Porto). To originate these cell lines, endothelial cells were isolated from the temporal lobe of brain of an adult female human with epilepsy and from an endothelioma in the cerebral cortex from mouse brain, respectively. Both cell types were cultured following the available cell line data sheet.

hCMEC/D3 cells were used between passages 25 and 35. Cells were seeded in cell culture flasks or plates coated with rat tail collagen type I (coating of the surfaces was

performed using a solution of rat tail collagen type I (Sigma) at a concentration of 150 µg.mL^{-1} during 1-2 hours at 37ºC. hCMEC/D3 cells were grown in EBM-2 medium (Lonza) supplemented with 5% Fetal Bovine Serum (FBS) (Gibco), 1% Penicillin-Streptomycin (Lonza), 1.4 µM of Hydrocortisone (Sigma), 5 µg.mL^{-1} of Acid Ascorbic (Sigma), 1% of Chemically Defined Lipid Concentrate (Gibco), 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Gibco) and 1 ng.mL^{-1} of bFGF (Sigma).

bEnd.3 cells were used between passages 25 and 30. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (1x) + GlutaMAX (Gibco) supplemented with 10% FBS (Gibco) and 1% Penicillin-Streptomycin (Lonza).

5. Gene expression – qRT-PCR

For Real-Time Polymerase Chain Reaction (qRT-PCR) analysis, both cell lines were used. Firstly, cells were incubated in the absence or presence of TTR variants for 24h (for analysis of TJs-related genes) or 8h (for analysis of angiogenesis-related genes), and total RNA was then isolated from cells using Trizol reagent (Invitrogen) following the instructions available in the product datasheet. RNA concentration and purity was quantified by reading the absorbance at 260 nm and the A260/A280 ratio, respectively, with NanoDrop photometer (Thermo Fisher Scientific), and RNA integrity was determined by running RNA samples on a 1% agarose gel stained with GreenSafe Premium (NZYtech). Then, 2 µg of RNA were first-reversed transcribed into cDNA using NZY First-Strand cDNA Synthesis kit (ZNYtech) and cDNA was saved at -20 ºC until used. Next, gene expression of tight junction-related genes and angiogenesis-related genes was evaluated by qRT-PCR. To evaluate a large set of genes related with tight junction proteins, a qRT-PCR was performed using a pre-designed PrimePCR Pathway Plate (Tight Junctions H96, BioRad), according to the manufacturer's protocol. Alternatively, primers were designed for some of the tight junction-related genes, according to their sequence available in the National Center for Biotechnology Information (NCBI) and using the functionality Primer-BLAST available in the same website. For the angiogenesis-related genes, human sequences available in the literature were used and the corresponding primers for mouse samples were designed as described above. Table 1

describes all primers designed in-house, whereas primers from the pre-designed PrimePCR Pathway Plate are not disclosed by the manufacturer.

Human or mouse GAPDH were used as an internal control for normalization. The reaction mix was done with the SYBR green reporter (iQ SYBR green supermix, BioRad) following the instructions presented by the manufacturer. PCR primers and conditions used for all analyzed genes are shown in table 1. The qRT-PCR was performed using the CFX96 Touch Real-Time PCR Detection System (BioRad). The relative quantification was performed according to the comparative method ($2^{-\Delta\Delta ct}$) (Livak and Schmittgen 2001).

Table 1. Sequences of the "in-house designed" primers. PCR condition was the same for all primers.

Cell line	Gene	Primer Sequence	PCR condition
hCMEC/D3	CLDN1	Forward: 5' CCCAGTCAATGCCAGGTACG 3' Reverse: 5' CAAAGTAGGGCACCTCCCAG 3'	Initial denaturation: 95ºC, 3 minutes Denaturating, annealing and extension (40 cycles): 95ºC, 15 seconds 60ºC, 30 seconds
	JAM-2	Forward: 5' CGCCCTGGGCTATCATAAGG 3' Reverse: 5' CAAAGGAGACACTCCGACCC 3'	
	CLDN3	Forward: 5'GACCAACCTGCATGGACTGT 3' Reverse: 5' TCAAGTATTGGCGGTCACCC 3'	
	CTNNB1	Forward: 5' GTCTGAGGAGCAGCTTCAGT 3' Reverse: 5' ACTTCAAATACCCTCAGGGGAAC 3'	
	CDH5	Forward: 5' CTTCACCCAGACCAAGTACACA 3' Reverse: 5' AATGGTGAAAGCGTCCTGGT 3'	
	VEGFR1	Forward: 5' CCCTCGCCGGAAGTTGTAT 3' Reverse: 5' GTCAAATAGCGAGCAGATTTCTCA 3'	
	VEGFR2	Forward: 5' ATTCCTCCCCCGCATCA 3' Reverse: 5' GCTCGTTGGCGCACTCTT 3'	
	ANGPT2	Forward: 5' AGGACACACCACGAATGGCATCTA 3' Reverse: 5' TGAATAATTGTCCACCCGCCTCCT 3'	
bEnd.3	VEGFR1	Forward: 5' GTGTCTATAGGTGCCGAGCC 3' Reverse: 5' CGGAAGAAGACCGCTTCAGT 3'	
	VEGFR2	Forward: 5' GCATACCGCCTCTGTGACTT 3' Reverse: 5' AAATCGCCAGGCAAACCCAC 3'	
	TGFB2	Forward: 5' AAAATCGACATGCCGTCCCA 3' Reverse: 5' ATGGCATCAAGGTACCCACA 3'	

6. Immunocytochemistry

hCMEC/D3 and bEnd.3 cells were grown on glass coverslips (Thermo Fisher Scientific) previously autoclaved. Cells were grown until reach 70% confluence and then incubated with their respective media in the absence or presence of TTR variants.

To study TJs protein levels, hCMEC/D3 cells were incubated with WT TTR (2 μM) or V30M TTR (4 μM) for 24h. For the study of collagen type IV levels, bEnd.3 cells were treated with WT TTR alone (2 μM) or with Aβ alone (10 μM) or with TTR and Aβ together, for 24h. Then, cells were washed with PBS and fixed with methanol for 15 min at RT. Following fixation, cells were permeabilized with 0.25% Triton X-100 in PBS for 10 min at RT. Next, blocking was performed with 5% BSA in PBS for 1h, followed by an overnight incubation with primary antibodies at 4ºC against the proteins of interest (table 2). After being washed with PBS, cells were incubated with the Alexa Fluor-488 donkey anti-rabbit IgG antibody (Invitrogen 1:1000) for 1h at RT. Coverslips were mounted with FluoroshieldTM with DAPI (Sigma-Aldrich). Visualization was done with the Zeiss Axio Imager Z1 microscope equipped with an Axiocam MR3.0 camera and Axivision 4.9.1 software.

Table 2. List of primary antibodies used in immunocytochemistry analysis.

Cell line	Protein	Primary antibody	Dilution
hCMEC/D3	Occludin	Polyclonal rabbit antibody anti-human occludin (Invitrogen)	1:12.5
hCMEC/D3	Claudin-3	Polyclonal rabbit antibody anti-human claudin-3 (Invitrogen)	1:12.5
bEnd.3	Collagen IV	Polyclonal rabbit antibody anti-mouse collagen IV (Abcam)	1:200

7. Wound Healing Assay

A wound healing assay was performed using both cell lines in a similar way. Cells were allowed to grow until confluence in a 12-well plate, and wounds were created in the

cell monolayer by scraping the plate with a 200 µl pipette tip. A single wash was performed to remove detached cells and media was then added in the presence or absence of TTR variants. Wounded monolayers were photographed immediately after wounding (t = 0h) and 6h, 8h and 10h later. The results were normalized for wound area at t= 0h and expressed as percentage of wound healing.

8. Animals

To evaluate collagen IV deposition in brain vessels, two mice models were used, an AD transgenic model and a non-transgenic model, both established in different TTR genetic backgrounds. The AD mouse model AβPPswe/PS1A246E/TTR transgenic mice was generated by crossing the AD mouse model AβPPswe/PS1A246E (Borchelt, Ratovitski et al. 1997) (B6/C3H background) purchased from The Jackson laboratory with TTR-null mice (TTR-/-) (SV129 background) (Episkopou, Maeda et al. 1993) as previously described (Oliveira, Ribeiro et al. 2011). As we intended to study the role of TTR in certain AD features, we used cohorts of littermates with different genetic backgrounds: AβPPswe/PS1A246E/TTR+/+ (carrying 2 copies of the TTR gene), AβPPswe/PS1A246E/TTR+/− (carrying 1 copy of the TTR gene) and AβPPswe/PS1A246E/TTR−/− (without TTR). In the next sections, the different genotypes AβPPswe/PS1A246E/TTR+/+, AβPPswe/PS1A246E/TTR+/-, AβPPswe/PS1A246E/TTR-/−, will be mentioned to as AD/TTR+/+, AD/TTR+/- and AD/TTR-/-, respectively. Tissues from these mice were already available in the lab from previous studies, and thus, these animals were not handled in the context of the current project. Brain tissue from AD/TTR+/- 7 months-old female mice treated with the TTR stabilizer iododiflunisal, also available in the lab from previous work (Ribeiro et al, 2014), were also analyzed in this work.

As stated, a non-transgenic mouse model containing different TTR backgrounds – TTR-wild type (+/+) and TTR-heterozygous (+/-) - was also studied. All mice were obtained from the same littermate as AD mice and were female aged 7 months. The different genotypes will be mentioned as $TTR^{+/+}$ (TTR-wild type) and $TTR^{+/-}$ (TTR-heterozygous).

Animals were housed in a controlled environment (12-h light/dark cycles, temperature between 22-24 °C, humidity between 45–65% and 15-20 air changes/hour), with

freely available food and water. All the above experiments were approved by the Institute for Research and Innovation in Health Sciences (i3s) Animal Ethics Committee and in agreement with the animal ethics regulation from Directive 2010/63/EU.

9. Tissue processing

TTR mice were anaesthetized with an intraperitoneal injection of a mixture of medetomidine (1 mg/kg) and ketamine (75 mg/kg). After anesthesia, the thorax was opened and it was exposed the heart. Then, it was placed a perfusion needle through the left ventricle into the ascending aorta and before start the perfusion, an incision in the right atrium was made. Firstly, PBS was perfused at a rate of 2 mL/min for 4 min, followed by perfusion of 4% paraformaldehyde (PFA) at a rate of 2 mL/min for 4 min. Following the perfusion, brains were removed and bisected longitudinally: each half of the brain was either frozen at -80 ºC for potential biochemical analysis and brain microvessel isolation, or fixed for 24h at 4ºC in 4% PFA. After, this last half brain was washed with PBS and then transferred to a 30% sucrose solution for cryoprotection before cryostat sectioning and immunohistochemical analyses.

10. Immunohistochemistry

To investigate collagen IV levels in brain vessels, 30 µm-thick coronal brain sections of mice were washed with PBS and dried O/N at RT on APES-precoated slides. Then, sections were permeabilized with 0.25% Triton X-100 in PBS for 10 min at RT, blocked with 5% BSA in PBS for 1h at RT and incubated with anti-collagen IV primary antibody (1:100) (Abcam) in 1% BSA in PBS overnight at 4ºC. Next, sections were washed with PBS and incubated with Alexa Fluor-568 donkey anti-rabbit IgG antibody for 1h at RT. To remove tissue autofluorescence, sudan black B solution (0.3% sudan black B in 70% ethanol) was applied for 5 min at RT, followed by washing one time with water and after with PBS. Then, brain sections were mounted with FluoroshieldTM with DAPI (Sigma-Aldrich) and visualization was done with the Zeiss Axio Imager Z1 microscope equipped with an Axiocam MR3.0 camera and Axivision 4.9.1 software. A total of fifteen randomly selected vessels in

the cortex of each mouse were photographed and the intensity of signal was measured using the ImageJ software.

11. Isolation and characterization of mouse brain microvessels

Isolation: Brain microvessels were isolated essentially as described previously (Qosa, Abuznait et al. 2012). Frozen brains from three mice were used. Briefly, brains were homogenized in Dulbecco's Phosphate-Buffered Saline (DPBS) with a Potter Tissue Grinder with 20 up-and-down strokes. To the homogenate, one volume of Ficoll 400 (30%) (Sigma) was added to a final concentration of 15%, and then centrifuged at 5000 rpm for 10 min at 4ºC. The resulting pellet was suspended in ice-cold DPBS containing 1% BSA and passed over a bead column. The bead column was made by cutting both ends of a 3-mL syringe, and one end was closed with a 70 micron cell strainer. Then, acid-wash glass beads (425-600 µm) (Sigma) were poured into the column extending to 1.5 cm from the filter. At this point, microvessels are attached to the beads and cell debris will be present in the flowthrough. The column was washed several times with DPBS and glass beads were transferred to a sterile 100 mm culture dish containing DPBS, followed by gently shaking the dish to separate microvessels from the beads. Finally, the microvessel suspension was centrifuged at 1000 g for 12 min at RT and the pellet resuspended in a small volume of DPBS.

Characterization: Firstly, some drops of hematoxylin were added to a small volume of microvessels suspension, and then placed in a microscope slide for further visualization. Microvessels were transferred to glass slide and let O/N at 37ºC to allow adhesion. Then, microvessels were washed with PBS, fixed for 15 min with 4% PFA at RT and permeabilized with 0,1% (v/v) Triton X-100 in PBS for 15 min. Blocking was performed with 1% BSA in PBS for 1 h, followed by incubation with primary antibodies at 4ºC against LRP1 (Abcam) and CD31 (Abcam) (both 1:100). After being washed with PBS, cells were incubated with the Alexa Fluor-488 donkey anti-rabbit and Alexa Fluor-568 donkey anti-mouse IgG antibody (Invitrogen 1:1000) for 1h at RT. Nuclei were stained with FluoroshieldTM with DAPI (Sigma-Aldrich). Visualization was done with the Zeiss Axio Imager Z1 microscope equipped with an Axiocam MR3.0 camera and Axivision 4.9.1 software.

12. Statistical Analysis

All quantitative data were expressed as mean value ± standard deviation (SD), in case of $n \geq 5$, data were expressed as mean ± standard error of the mean (SEM). qRT-PCR and wound healing assay results were statistically analyzed for significant difference using two-tailed Student's t-test. Differences in brain collagen IV levels were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test when three different mice genotypes were compared or Student's t-test when only two different mice genotypes were compared. p-values lower than 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism 6 software for Windows.

Results

1. Effect of TTR on tight junction-related genes

1.1. At the transcript level

As referenced in the introduction, TTR is capable to modulate important genes of BBB such as LRP1, but there are no published studies on the effect of TTR on TJs, which are extremely important components of the BBB. As a first approach, we evaluated the transcription level of several tight junction-related genes in hCMEC/D3 cells using a PCR array, since it allows to analyze changes in gene expression of numerous genes at the same time.

With this objective, changes in the transcript level were investigated by comparing hCMEC/D3 cells incubated in the absence or presence of WT TTR, at the final concentration of 2 µM for 24h. We choose this condition because it was previously used by authors to study the modulation role of TTR in gene expression (Alemi, Gaiteiro et al. 2016). Alterations in gene expression are presented as the ratio between WT-treated cells and control (absence of TTR).

As shown in figure 15, qRT-PCR results indicate that TTR induced alterations in gene expression of several genes. The most pertinent alterations obtained were the up-regulation of JAM-2 and CLDN1, and down-regulation of CLDN2 and TJP3. Curiously, these results

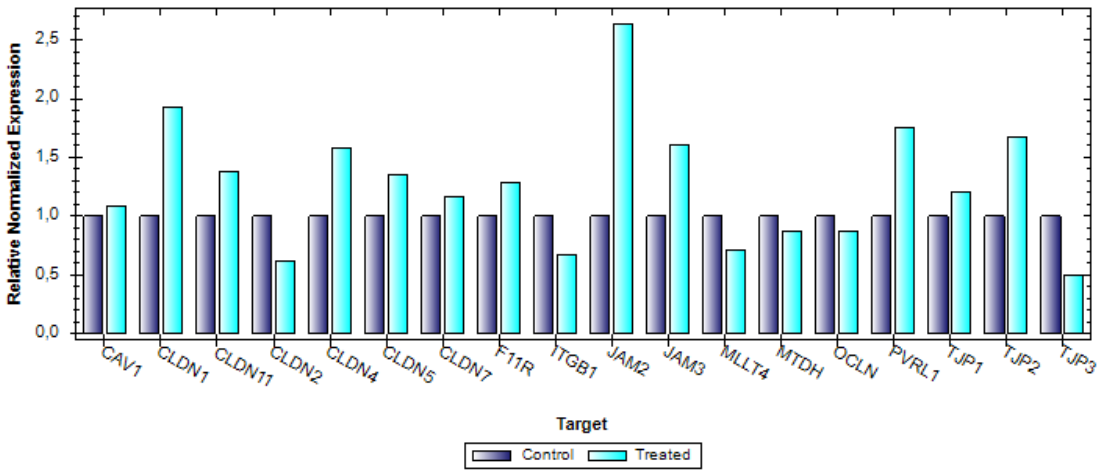

Figure 16. PCR array analysis of gene expression of several TJs-related genes. A PCR array was used to characterize endothelial cells response to WT TTR. hCMEC/D3 were incubated with or without WT TTR at the final concentration of 2µM for a period of 24h. Data represent the normalized gene expression of each target in TTR-treated cells relative to the control. A "pool" of cDNA constituted by 3 different cDNAs was used.

suggest that TTR alters the gene expression of different genes of the same family in a different way, such as the CLDN family. It is also possible to observe that numerous genes present the same transcript level with or without TTR.

Although arrays are a good tool to study global transcriptional alterations, we do not have knowledge of the sequences of the primers used. Thus, we decided to confirm the results for the two most up-regulated genes, JAM-2 and CLDN1, by using primers designed by us.

Surprisingly, the results obtained using the commercial PCR array were not validated for both genes, since no differences in gene expression of CLDN1 and JAM-2 were verified (figure 16A).

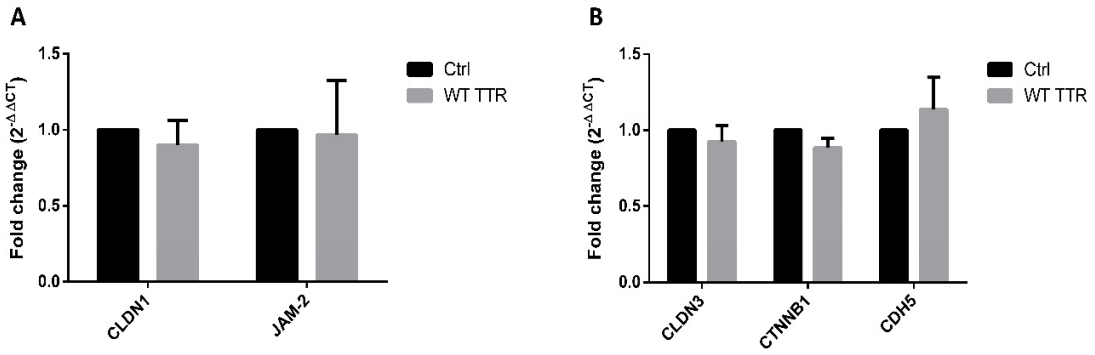

Figure 17. qRT-PCR analysis of TJs-related genes using "in-house" primers. hCMEC/D3 cells were incubated with or without TTR (2 µM) for 24h. (A) Transcript levels of CLDN1 and JAM-2 were not altered by the presence of TTR, not validating the results obtained using the PCR array. (B) CLDN3, CTNNB1 and CDH5 did not show differences in gene expression at the transcript level with TTR incubation. Data represent the –fold change in gene expression of each target in TTR-treated cells relative to the control.

Moreover, we analyzed the gene expression of three other genes, CLDN3, CTNNB1 and CDH5, which are also important in tight junction formation, and were not evaluated in the commercial PCR array. As observed in figure 16B, analysis by qRT-PCR showed no significant differences for all these three genes.

1.2. Effect at the protein level

It is commonly known that there is a low correlation between mRNA level and its coding protein level, despite being reasonable to see high protein level with high mRNA level (or the opposite case).

In this sense, we decided to further confirm if TTR has no effect in tight junction-related genes expression by performing immunofluorescence analysis, verifying protein levels of two important tight junction proteins, OCLN and CLDN3, which were analyzed in the PCR array and individual qRT-PCR, respectively.

Similarly to the previous study of gene expression at transcript level, protein level was investigated by comparing hCMEC/D3 cells incubated in the absence or presence of WT TTR, at the final concentration of 2 µM for 24h. Moreover, we also incubated cells with V30M TTR at the final concentration of 4 µM for 24h to evaluate if this variant associated with disease could change CLDN3 protein level.

Immunofluorescence results demonstrated that WT TTR did not change the protein level of both proteins (figure 17), corroborating the results obtained at the transcript level, where no significant differences were obtained for these proteins. Also, V30M TTR was not capable to affect gene expression at the protein level of CLDN3.

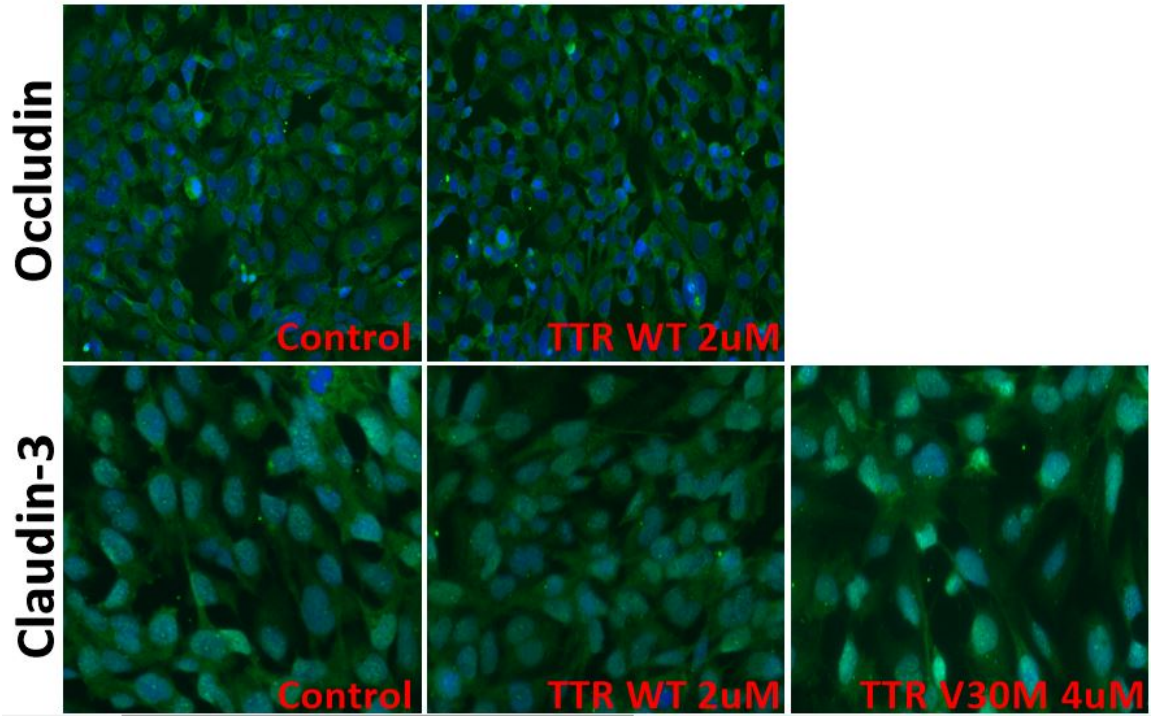

Figure 18. Analysis of protein level of tight junctions in hCMEC/D3 cells without or with incubation of TTR for 24h. Immunofluorescence analysis of Occludin and Claudin-3 expression in hCMEC/D3 cells stained with an antibody against each protein (green). Nucleus of cells are stained with DAPI (blue).

Altogether, these observations indicate that TTR has no effect in gene expression of tight junction-related genes in hCMEC/D3 cells, both at the transcript and protein levels, in the conditions and cell type used.

2. Effect of TTR on the angiogenic capacity of endothelial cells

A previous study, using HUVEC, explored the biological effect of TTR variants on angiogenesis (Nunes, de Oliveira et al. 2013). In this work, authors compared the effect of WT TTR vs V30M TTR, concluding that the two TTR variants induced a different response with regard to the angiogenic capacity, since in the presence of V30M TTR, cells expressed less the pro-angiogenic genes as compared to cells incubated with WT TTR. In this work, however, researchers did not evaluate if TTR per se is important for regulation of angiogenesis, i.e. comparison between the presence of WT TTR and cell culture media alone was not performed.

2.1 Effect of TTR variants in the expression of pro-angiogenic genes in hCMEC/D3 cells

Following what was previous said, we designed our study aiming at evaluating the role of TTR in brain angiogenesis. In order to understand it, we examined the expression of genes known to be important in angiogenesis, such as VEGFR1, VEGFR2 and ANGPT2. For this, we incubated cells in the absence or presence of WT TTR (4 µM) and V30M TTR (4 µM) for 8h, replicating the conditions used by the authors of the previous study (mentioned above), but using the hCMEC/D3 cell line.

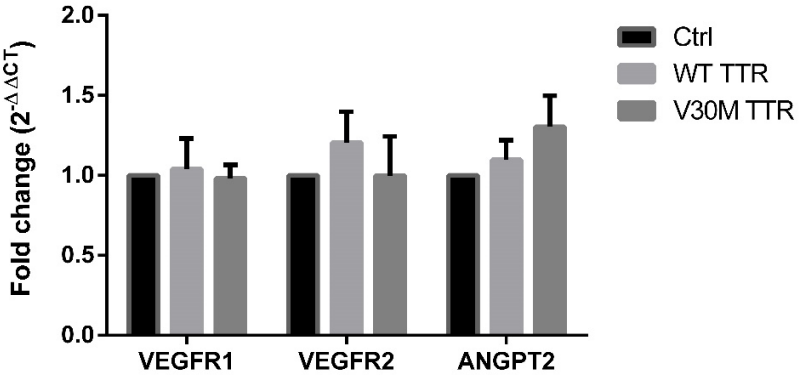

Figure 19. qRT-PCR analysis of pro-angiogenic genes in hCMEC/D3 cells. Cells were incubated with or without TTR variants for 8h at a final concentration of 4 µM. Transcript levels of three different pro-angiogenic genes are presented. Data represent the –fold change in gene expression of each target in TTR-treated cells relative to the control.

According to our qRT-PCR results, the expression of such genes, at the transcript levels, did not change at 8 h (figure 18), and thus, similar levels were found in the absence or presence of TTR variants. These results indicate neither variant modulated the expression of these angiogenesis-related genes, neither positively nor negatively. Importantly, we also did not see a difference in the gene expression between WT TTR and V30M TTR. It is important to highlight it, since Nunes and colleagues verified a down-regulation of these three genes with V30M TTR, comparing to WT TTR.

2.2 Effect of TTR variants in the hCMEC/D3 cells migration capacity.

Moreover, and to confirm that TTR is not involved in angiogenic-related events, we also evaluated migration, an essential part of the angiogenesis process. For that, hCMEC/D3 cells were cultured until confluent, followed by scraping the monolayer, initiating the wound process. Then, hCMEC/D3 cells were exposed to different TTR variants (WT, V30M and L55P) at the final concentration of 4 µM. As observed in figure 19, the ECs migratory response was similar between control and all TTR variants, since cells incubated in the absence or presence of TTRs achieved similar wound area at 8h, meaning that independently on the TTR variant, the presence of TTR does not alter the capacity of cells to migrate. Similarly, the presence of WT TTR at different concentrations did not influence hCMEC/D3 cell migration (figure 20).

2.3. Impact of TTR variants in the migration capacity of bEnd.3 cells

Altogether, our results do not agree with the ones from two previous studies (Nunes, de Oliveira et al. 2013, Shao and Yao 2016). It is important to note that some experimental conditions were not the same which can account for the observed differences. For example, different batches of TTR can influence the results. Other important experimental difference is the cell lines used by both authors (HUVECs and hRECs) compared to ours (hCMEC/D3).

In order to explore the influence of the cell line in results, we used another cell line, the bEnd.3 cell line.

As shown in figure 21, ECs migrated in a similar velocity, independently on the absence or presence of TTR. However, importantly, bEnd.3 cells do not appear appropriated

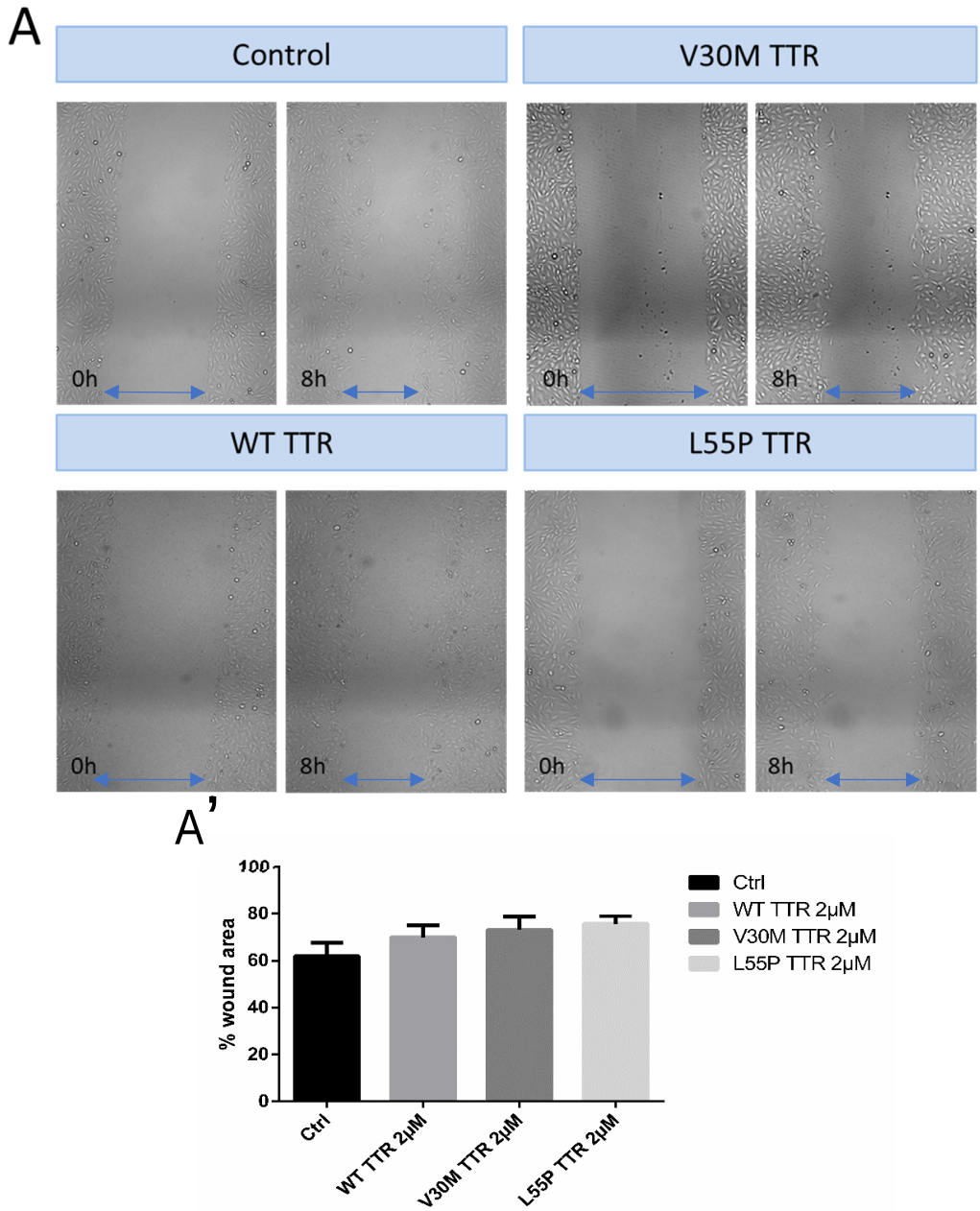

Figure 20. Effect of TTR variants on the migration of hCMEC/D3 cells. (A) hCMEC/D3 cells were cultured until confluent, and the monolayer was scraped with a 200 µl pippete tip to initiate wounding, following addition of TTR variants. Monolayers were followed over time and the wound area was measured at t = 0h and t = 8h. (A') Quantification of results for each treatment was recorded and the means ± S.D. were calculated after normalizing for wounded area at t = 0h and expressed as percentage of wound area.

for migration studies at short times, since its migration happens very slowly – in our results, cells closed less than 10% of the wound during 10h -, being difficult to observe a possible effect. Despite it, we advance to the analysis of three angiogenesis-related genes by qRT-PCR. Once again, no significant differences were observed between cells incubated with or without WT TTR (figure 21B).

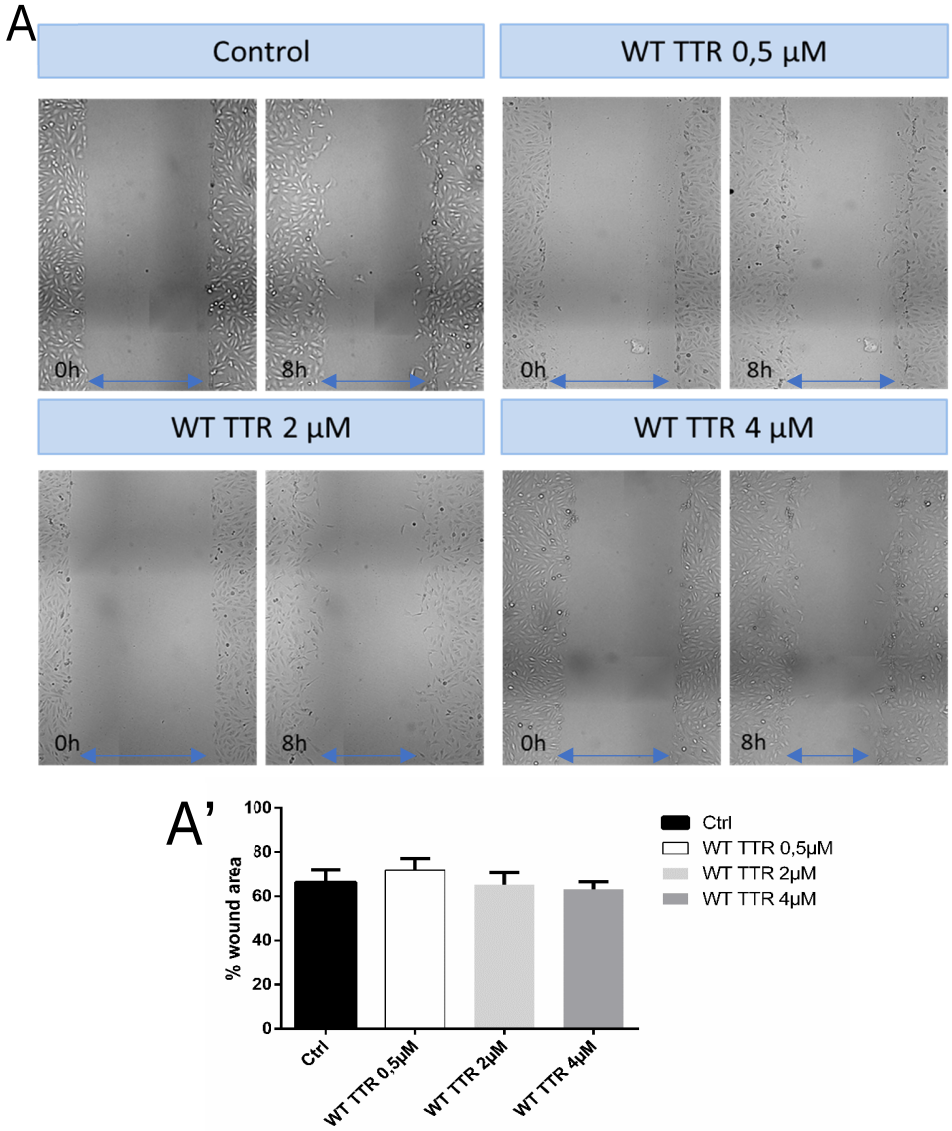

Figure 21. Effect of WT TTR concentration on the migration of hCMEC/D3 cells. (A) hCMEC/D3 cells were cultured until confluent, and the monolayer was scraped with a 200 µl pippete tip to initiate wounding, following addition of WT TTR at different final concentrations. Monolayers were followed over time and the wound area was measured at t = 0h and t = 8h. (A') Quantification of results for each treatment was recorded and the means ± S.D. were calculated after normalizing for wounded area at t = 0h and

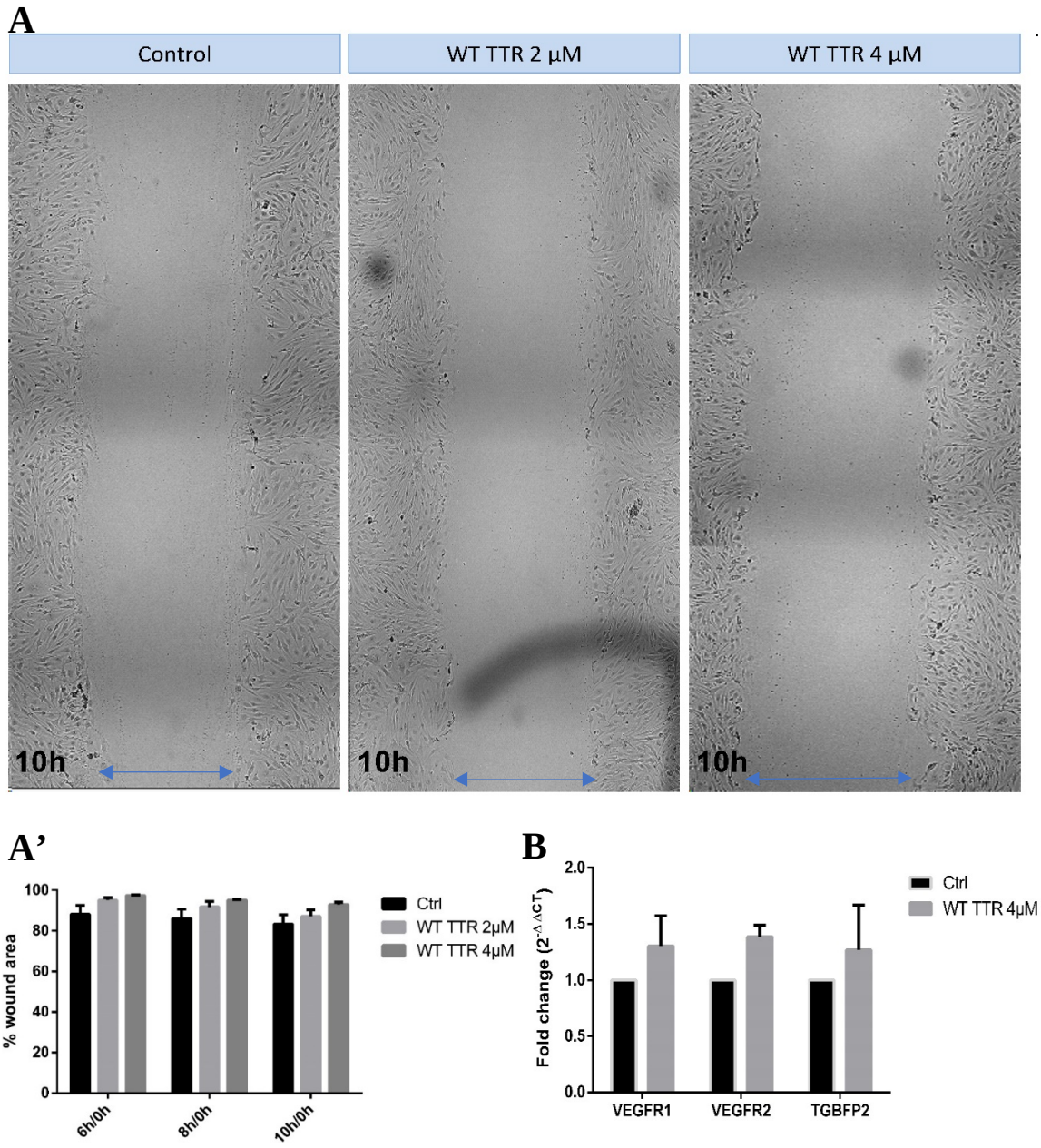

Figure 22. Effect of WT TTR on the angiogenic capacity and on gene expression of pro-angiogenic genes in bEnd.3 cells. (A) bEnd.3 cells were cultured until confluent, and the monolayer was scraped with a 200 µl pippete tip to initiate wounding, following addition of WT TTR at two different final concentrations. Monolayers were followed over time and the wound area was measured at t = 0h, t = 6h, t = 8h and t = 10h. (A') Quantification of results for each treatment was recorded and the means ± S.D. were calculated after normalizing for wounded area at t = 0h and expressed as percentage of wound area. (B) Transcript levels of three different pro-angiogenic genes were analyzed. Data represent the –fold change in gene expression of each target in TTR-treated cells relative to the control.

3. Effect of TTR in collagen IV levels in AD

The BM is an important element of the neurovascular unit, and acts as a mechanical barrier. Moreover, it is also essential to maintain BBB integrity and regulate angiogenesis. Several studies have already demonstrated that collagen IV, an important protein of BM, is increased in brain vessels of AD, both in patients and mice, compared to non-AD, suggesting that Aβ can be involved in this increase: These greater levels create a thick barrier, which can lead to an impairment of Aβ clearance by blocking its removal.

Since TTR levels are decreased in AD, we asked whether there is a relationship between the decrease in TTR and the increase in the collagen layer.

Thus, our first approach consisted in evaluating collagen IV levels in brain vessels of AD/TTR female mice 7 months old with different TTR backgrounds: AD/TTR$^{+/+}$, AD/TTR$^{+/-}$ and AD/TTR$^{-/-}$. Collagen IV levels were determined using immunohistofluorescence. As shown in figure 22, collagen IV levels are significantly increased in microvessels from AD/TTR$^{+/-}$ and AD/TTR$^{-/-}$ mice, compared to AD/TTR$^{+/+}$, where AD/TTR$^{+/-}$ mice presented the highest amount of collagen IV. Although these results suggested that TTR influenced the thickness of the basement membrane, in particular the collagen IV layer, we could not determine if the effect was direct or indirect. It is very well established that TTR binds Aβ avoiding its aggregation and toxicity and promoting its elimination from the brain. One hypothesis is that high levels of Aβ, as it happens in AD, either due to increased production, reduced elimination or both could be responsible for the increase in collagen IV. As such, it is possible that AD/TTR+/- and AD/TTR-/- mice show increased amount of collagen IV because less TTR is available to interact with Aβ. Thus, in order to unravel this question, we firstly compared collagen IV levels in non-transgenic mice with two different TTR backgrounds, TTR$^{+/+}$ and TTR$^{+/-}$, allowing to understand if TTR is directly involved.

Immunohistofluorescence results (Figure 23) revealed that decreased levels of TTR did not originate modifications in collagen IV levels in brain vessels. This result led us to conclude that TTR does not directly influence collagen IV, and most likely the results obtained with AD/TTR+/- and AD/TTR-/- mice indeed relate to the fact that less TTR is available to sequester Aβ peptide and to avoid its aggregation in the brain.

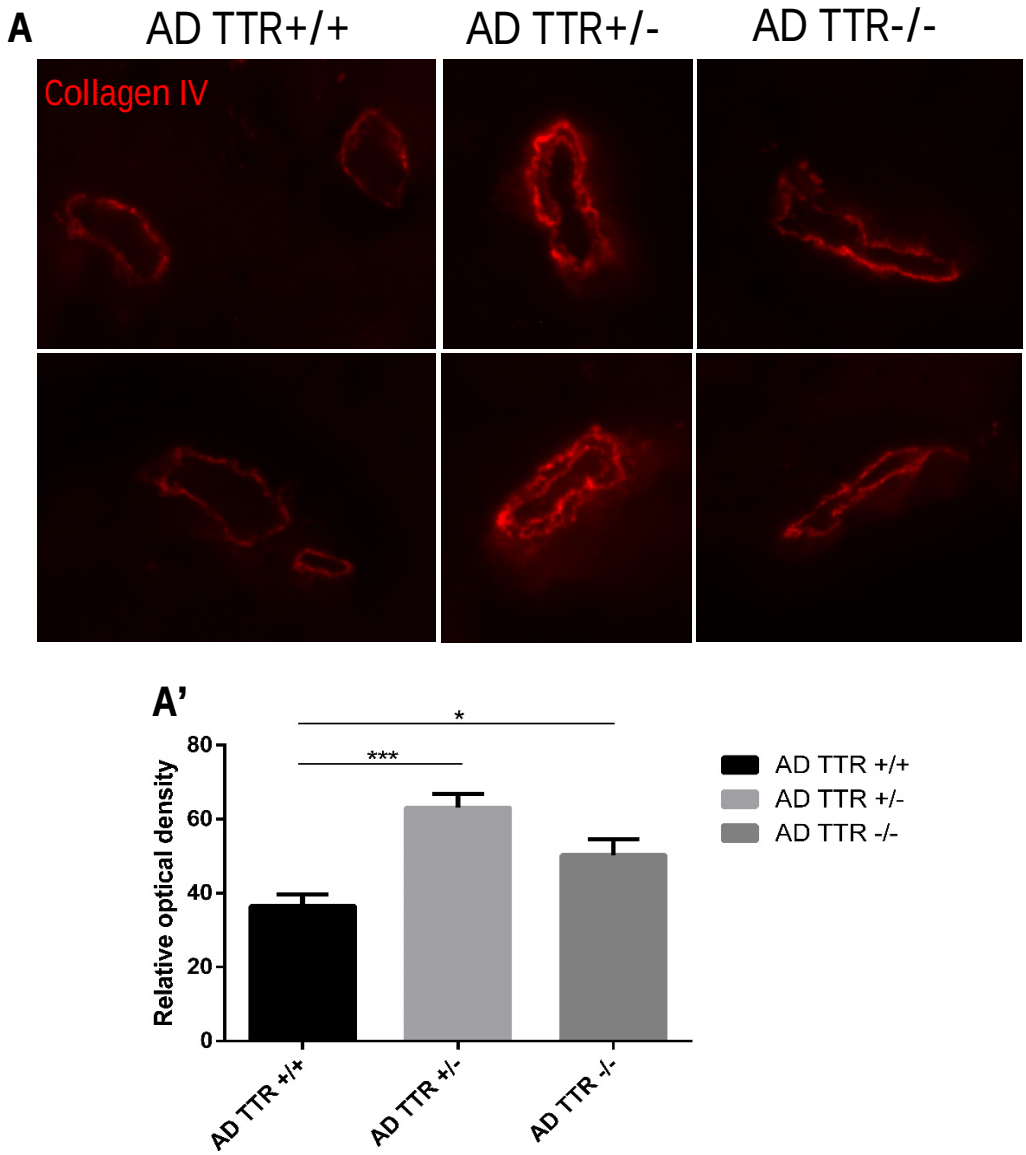

Figure 22. Effect of TTR genetic reduction in collagen IV levels in AD mice. (A) Representative images of different vessels derived from AD mice with different TTR genetic backgrounds. Collagen IV levels (red) were evaluated by immunohistofluorescence with an anti-collagen IV antibody in 7-month-old AD $TTR^{+/+}$ (n=7), AD $TTR^{+/-}$ (n=7) and AD $TTR^{-/-}$ female mice (n=3). (A') Quantification of collagen IV levels, showing its significantly increased levels in microvessels from AD TTR+/- and AD TTR-/- mice compared to AD TTR+/+ mice, where AD TTR+/- mice presented the highest amount of collagen IV. Values shown as means ± SEM. *p<0,05; ***p<0,001.

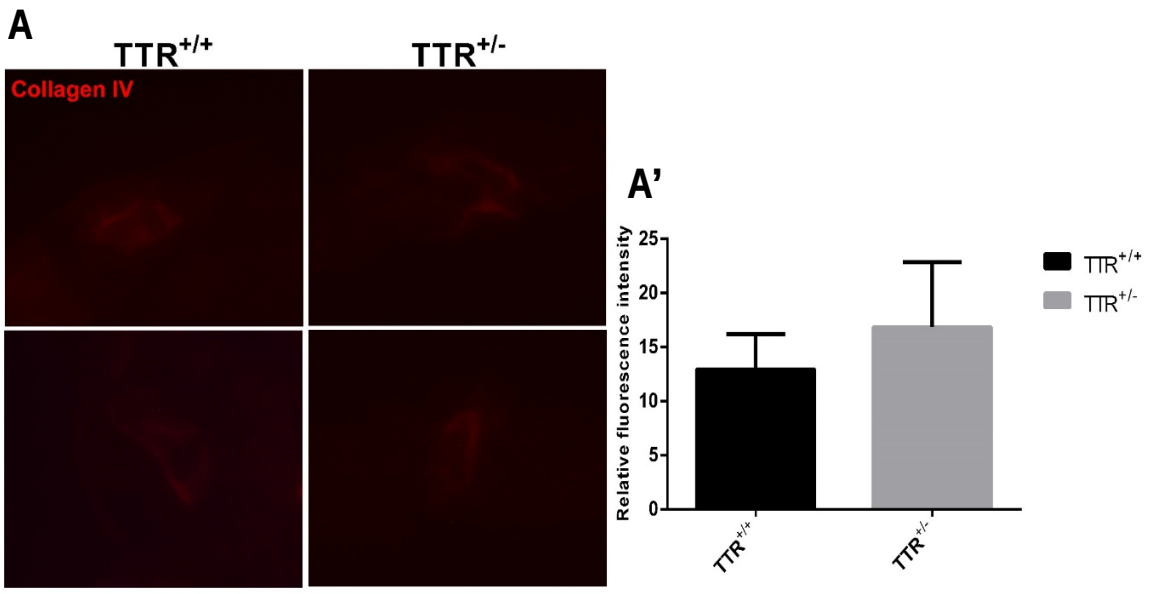

Figure 23. Effect of TTR genetic reduction in collagen IV levels in brain vessels. (A) Representative images of different vessels derived from mice with different TTR genetic backgrounds. Collagen IV levels (red) were evaluated by immunohistofluorescence with an anti-collagen IV antibody in 7-month-old $TTR^{+/+}$ (n=3) and $TTR^{+/-}$ (n=3) (A') Quantification of collagen IV levels, showing no significant differences in microvessels from $TTR^{+/+}$ and $TTR^{+/-}$. Values shown as means ± S.D.

Following our hypothesis, we first decided to investigate if different Aβ species (soluble, oligomers and fibrils), can lead to an increase of collagen IV levels. Thus, we evaluated collagen IV levels in bEnd.3 cells incubated with 10 µM of each Aβ specie for 24 hours. The presence of different Aβ species was confirmed by transmission electron microscopy (Figure 24). As it possible to observe, soluble Aβ solution presents already some aggregates (figure 24A). Preparation of a solution containing 100% of soluble Aβ using synthetic $A\beta_{42}$ is highly challenging and nearly impossible. However, oligomers preparation (figure 24B) presented a high amount of Aβ oligomers and aggregates with different sizes. The fibrils preparations (figure 24C), which are very distinct from the other species,

presented the characteristic fibrillary structure with different lengths, but still with some oligomers.

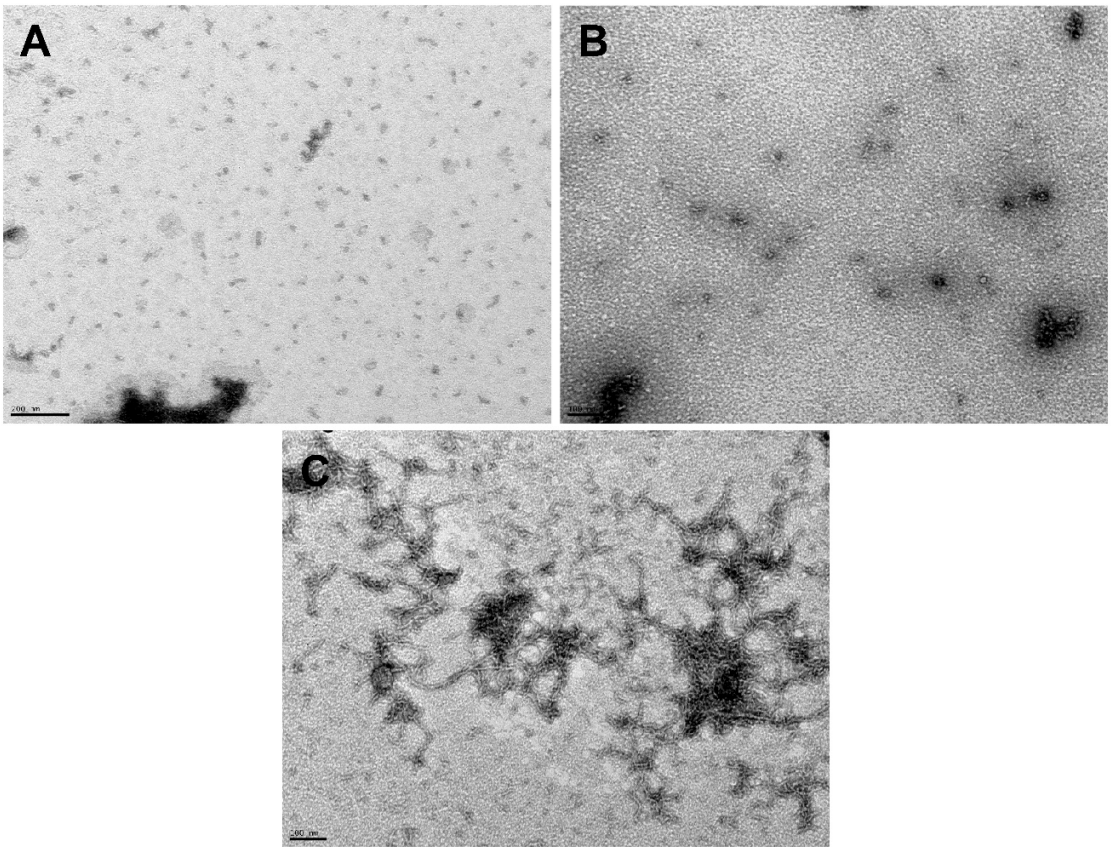

Figure 24. Morphological characterization of Aβ species by TEM analysis. Visualization of (A) Soluble Aβ, (B) Oligomers and (C) Fibrils using TEM.

Immunofluorescence results (figure 25) reveal that, in fact, Aβ leads to an increase of collagen IV levels, supporting our hypothesis. Moreover, the increase of collagen IV is independent on the Aβ specie, since no differences between species were observed. However, due to the difficulty in obtaining soluble, monomeric Aβ, care must be taken when drawing such conclusions.

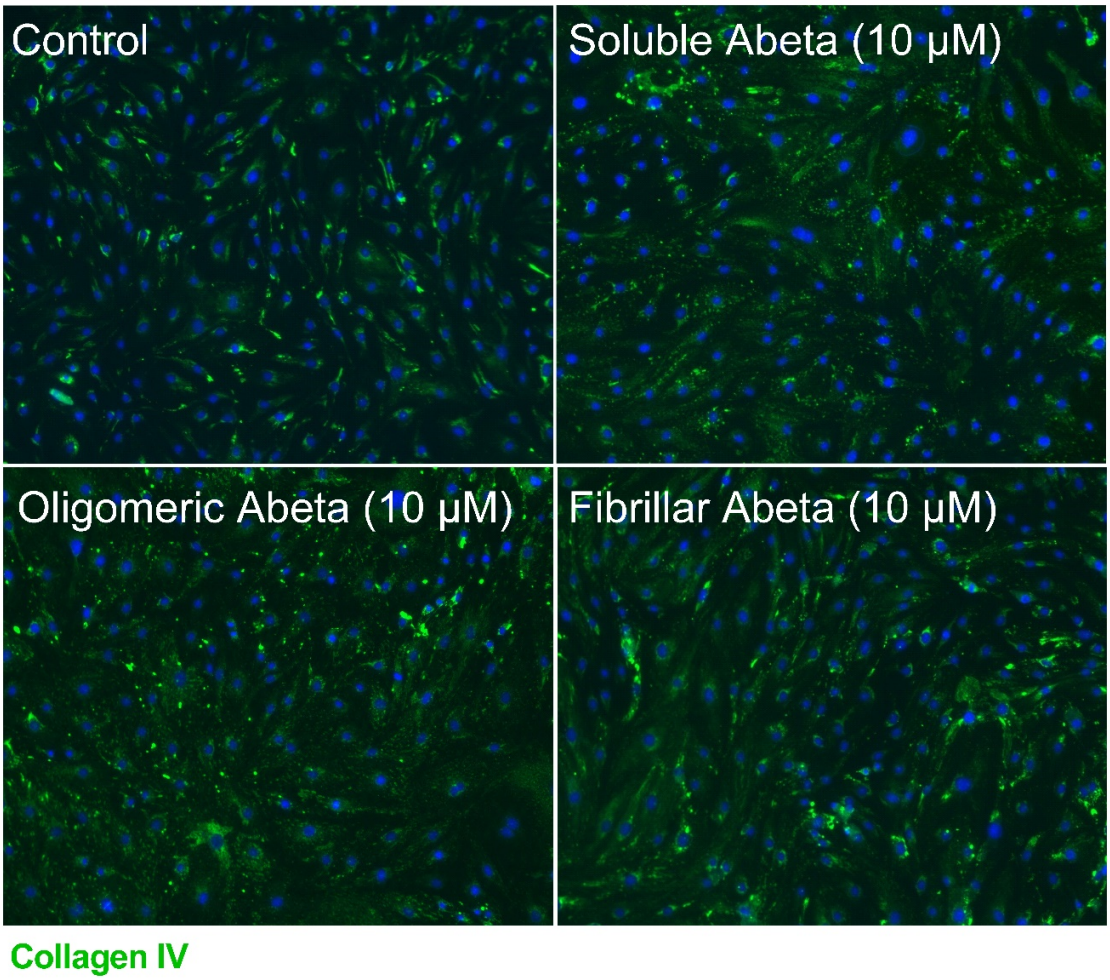

Figure 25. Analysis of collagen IV levels in bEnd.3 cells without or with incubation of Aβ species for 24h. Immunofluorescence analysis of Collagen IV expression in bEnd.3 cells stained with an antibody against our target (green). Nucleus of cells are stained with DAPI (blue).

To ascertain if TTR exerts a neuroprotective role in the decrease of collagen IV levels by decreasing Aβ effects through its clearance, we evaluated if the simultaneous presence of TTR and Aβ results in the preservation/normalization of collagen IV levels. Thus, we incubated bEnd.3 cells with TTR alone, soluble Aβ alone or both together for 24 h.

Visualization of immunofluorescence results (figure 26) shows that Aβ leads to an increase of collagen IV levels (as also shown in figure 25), while TTR didn't alter the amount of this BM's protein. Interestingly, when Aβ and TTR were incubated simultaneously, no differences in collagen IV amount were observed.

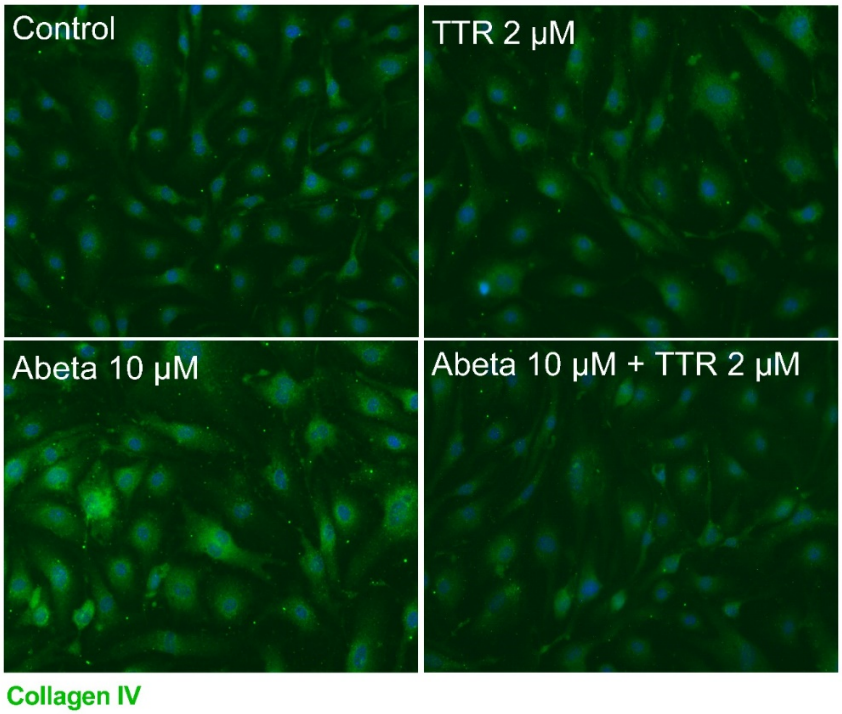

Figure 26. Analysis of collagen IV levels in bEnd.3 cells with incubation of TTR alone, Aβ alone or both together. Immunofluorescence analysis of Collagen IV expression in bEnd.3 cells stained with an antibody against our target (green). Nucleus of cells are stained with DAPI (blue).

Work developed in our lab has previously indicated that TTR stability is impaired in AD; stabilizing TTR with small chemical compounds known to stabilize its tetrameric fold was then suggested as a therapeutic avenue in AD. Administration of IDIF to AD mice, a potent TTR stabilizer, resulted in amelioration of some AD features, such as the cognitive function and decreased Aβ brain levels. In this work, we evaluated the collagen IV layer in AD/TTR+/- animals, non-treated versus IDIF-treated. As depicted in figure 27, AD/TTR$^{+/-}$ mice treated with IDIF presented a significant decrease in collagen IV levels, presenting new evidence for the involvement of TTR.

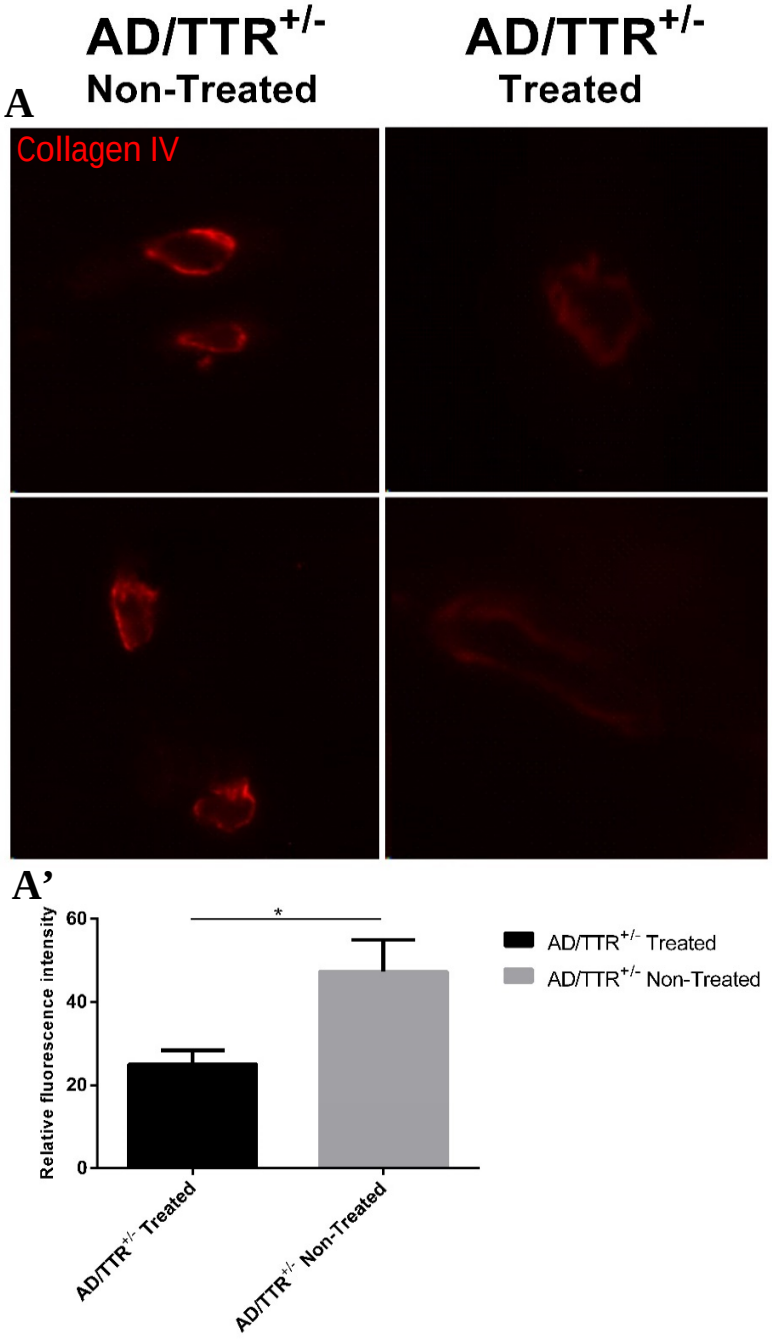

Figure 27. Effect of IDIF treatment in collagen IV levels of AD/TTR$^{+/-}$ mice brain. (A) Representative images of different vessels derived from AD/TTR$^{+/-}$ mice treated without or with IDIF. Collagen IV levels (red) were evaluated by immunohistofluorescence with an anti-collagen IV antibody in 7-month-old AD/TTR$^{+/-}$ non-treated (n=7) or treated (n=7) with IDIF (A') Quantification of collagen IV levels, showing significant difference in collagen IV levels, where treated mice presented a decrease in the amount of collagen IV. Values shown as means ± S.D *p<0.05

Our results suggest that Aβ, in the absence of TTR or its reduced levels, can induce changes in collagen IV, leading to its increase and thickening of the BM. We speculate that, in turn, this will prevent Aβ from being cleared and reaching the blood, further contributing to increased brain Aβ levels, resulting in its accumulation and aggregation in the brain.

4. Isolation of brain microvessels

Several studies in our group examined the effects of TTR in cultured brain endothelial cells, which have yielded important data about BBB. However, cell culture of endothelial cells are a very simple model, since it is known that a complex crosstalk between different cells from the NVU is essential for a correct development and maintenance of BBB. Thus, we intended to perform new studies (as mentioned in future perspectives) using brain microvessels, a way to use an intact BBB. For this, we tested some protocols describing techniques for isolating microvessels from mice brain. As observed in figure 28, structurally intact microvessels were successfully isolated. Samples presented cells morphologically

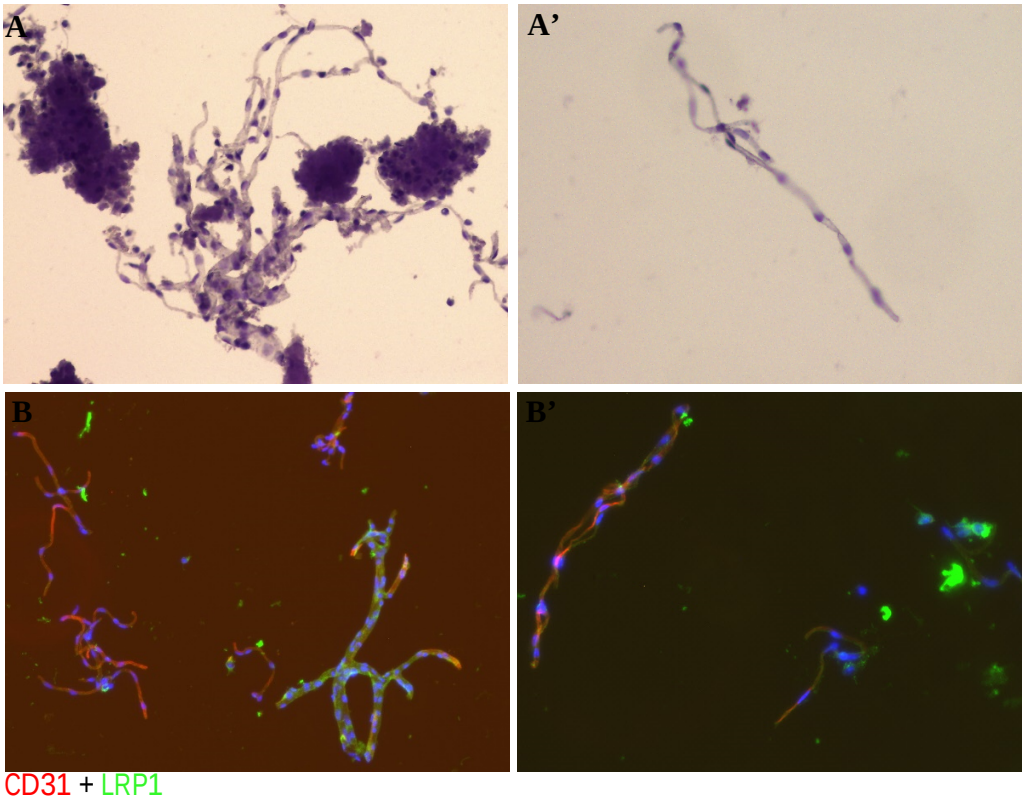

Figure 28. Representation of brain microvessels obtained from mice. (A and A') Microvessels stained with hematoxylin and (B and B') showing positive staining for LRP1 (Green) and CD31 (Red).

resembling microvessels, presenting different sizes and arrangements (isolated or in branches). Expression of LRP1 and CD31, characteristic proteins of vascular endothelial cells, was found in all microvessels. However, as seen in all images, samples presented relatively low purity, since a high number of microvessels are covered by adherent cells. Further optimization of the protocol should be done, in order to improve the microvessel purity of our samples.

Discussion

The blood-brain barrier is a special membrane barrier found in the brain vessels, allowing a physical separation between the blood and brain. This barrier is enabled by the presence of an important feature between endothelial cells, the so called tight junctions. In addition, several other members of the neurovascular unit and the cross-talk between them are identified and known to be important for BBB regulation and maintenance (Abbott, Patabendige et al. 2010).

Changes in the BBB are associated with the disease, which together with the challenge created by BBB's ability to limit access to many substances, including CNS therapeutic compounds, makes BBB a target for many studies (Banks 2016). In fact, several diseases have the disruption of the BBB as an important characteristic, such as stroke, Parkinson's disease and Multiple Sclerosis (Obermeier, Daneman et al. 2013). AD, the most prevalent form of dementia, is another example. In AD, in addition to a decrease of tight junction levels, several Aβ transporters which regulate its influx and efflux are altered, favoring disease. RAGE, the main influx receptor of Aβ is increased, while LRP1 and P-gp, important efflux transporters are decrease, leading to an increase of Aβ in the brain, contributing for the progress of AD (Marques, Sousa et al. 2013).

Previous studies have shown transthyretin as a neuroprotective molecule in AD, mainly describing its important function as an Aβ carrier, contributing to Aβ clearance at the BBB (Alemi, Gaiteiro et al. 2016). This is especially important, since it is thought that impaired Aβ clearance is one of the most important features which lead to sporadic AD, the large majority of AD cases. Besides its role as a neuroprotective protein in disease, recently studies have shown TTR as a CNS gene modulator in physiology. It was found that TTR is capable to promote a robust neurite outgrowth response in neurons by its interaction with megalin (Gomes, Nogueira et al. 2016) and also induce transcription of IGF-IR in neurons (Vieira, Gomes et al. 2015). In endothelial cells, TTR is also capable to increase the levels of LRP1 (Alemi, Gaiteiro et al. 2016), an important finding for AD pathology, since LRP1 is decreased in this disease.

Previous work in the lab indicated that occludin levels, decreased in the presence of Aβ, were normalized in cells co-incubated with Aβ and TTR, and thus we started by evaluating the effect of exogenous TTR in the expression of TJs-related genes, in hCMEC/D3 cells. However, we did not observe any effect of TTR in the genes evaluated, including

occludin, and thus we concluded that the initial observation on the effect of TTR in occludin is mostly likely a reflex of the direct action of TTR in Aβ, decreasing its toxicity. It is important to highlight the lack of studies on this subject, not allowing us to compare the data obtained in our work. Nevertheless, in the future, it would be important to evaluate other aspects of the TJs, such as localization and structure, using techniques such as electron microscopy, which enable detailed studies on cellular morphology.

With regard to TTR modulating ability, Nunes et al found that TTR regulates angiogenesis by conferring different molecular identities to umbilical endothelial cells (Nunes, de Oliveira et al. 2013), although, in their work, the authors compared the effect of WT TTR and V30M, without relating with the results obtained in the absence of TTR. Nevertheless, they found that V30M TTR decreased the expression of pro-angiogenic genes, as compared to the WT counterpart, in HUVEC cells. Thus, we investigated whether TTR variants had any modulatory function in angiogenic-related genes, VEGFR1, VEGFR2 and ANGPT2, using hCMEC/D3 cells, or in VEGFR1, VEGFR2 and TGFBP2 using bEnd.3 cells. Our results revealed that TTR variants did not alter the transcript levels of any of analyzed genes in both hCMEC/D3 and bEnd.3 cells. Moreover, to complement our qRT-PCR results, we evaluated endothelial cell migration, an important step of angiogenesis, using an in vitro wound healing assay, in the presence of WT, V30M and L55P TTR. We found no effect of any of the variants, nor positive, nor negative as compared to the absence of TTR, contrarily to Nunes and co-workers that reported V30M TTR as decreasing the healing capacity of HUVEC cells, compared to WT TTR. It is important to highlight that bEnd.3 cells or the experimental conditions used did not appear appropriated for migration studies in our time condition, since in our results, cells closed less than 10% of the wound during 10h, indicating its migration happen very slowly. Nevertheless, we expected a negative effect at least for the amyloidogenic variants V30M and specially the L55P. While V30M TTR is the most common TTR mutation in FAP patients, in Portugal, and results in an unstable tetramer that tends to dissociate into monomers, resulting into protein aggregation, accumulation and amyloid formation, L55P TTR is a very aggressive mutation, resulting in a very severe phenotype. FAP is a progressive, neurodegenerative disease, with onset in the adult life, and probably our model does not mimic these essential characteristics

of FAP. In an attempt to surpass this limitation, future experiments should assess the effect of higher TTR concentrations.

In AD, several works report on the thickening of the BM, namely on the layer of collagen IV which can act as a barrier in brain vessels, preventing Aβ passage from the brain to the blood and therefore, also becoming responsible for Aβ accumulation (Uspenskaia, Liebetrau et al. 2004, Tian, Shi et al. 2006). Since TTR is involved in Aβ elimination from the brain, we analyzed collagen IV levels in brain vessels of AD mice with different backgrounds of TTR, concluding that AD/TTR$^{+/-}$ and AD/TTR$^{-/-}$ mice presented high levels of collagen IV surrounding brain vessels compared to AD/TTR$^{+/+}$ mice. However, in our AD/TTR model, TTR levels were not totally inversely proportional to collagen IV levels, since AD/TTR$^{-/-}$ didn't present higher levels of collagen than AD/TTR$^{+/-}$. As explained in the characterization of this animal model (Oliveira, Ribeiro et al. 2011), the detrimental effects of the genetic reduction of TTR in AD/TTR$^{+/-}$ female mice are not always observed in AD/TTR$^{-/-}$ mice, such as increased levels of Formic acid- soluble $A\beta_{42}$ and decreased levels of brain 17β-estradiol, being the later a hormone capable to induce TTR expression (Oliveira, Ribeiro et al. 2011). One hypothesis is that AD mice without any TTR allele might undergo transcriptional remodeling through which compensatory mechanisms can occur, overcoming the possible effects of the total lack of TTR in the pathogenesis of AD. Nevertheless, our results indicate that a decrease in TTR leads to an increase of collagen IV, probably indirectly as no differences in collagen IV were found between non-transgenic mice TTR+/+ and TTR+/-. We then hypothesized that high levels of Aβ, as it happens in AD, either due to increased production, reduced elimination or both can be responsible for the increase in collagen IV. Since the initial characterization of the AD/TTR model used in the current study showed that Aβ brain burden is also higher in AD/TTR+/- than in AD/TTR+/+, this could further explain the increase in collagen IV layer. Thus, TTR is acting indirectly on collagen IV levels, decreasing this Aβ effect, by promoting its clearance. In order to test this hypothesis, we performed cellular studies and showed that indeed Aβ induced increased collagen IV in bEnd.3 cells which was slowed down by TTR, probably by binding the peptide and avoiding its aggregation and accumulation. We also attempted to clarify which Aβ species is capable of inducing collagen IV expression, the soluble monomer, the oligomers and/or the fibrils. However, we failed at obtaining pure populations of each of these species,

as demonstrated by TEM, and all of them had a positive effect in collagen IV levels. Thus, in the future, it is necessary to perform the experiment with homogeneous populations of Aβ species, allowing to distinguish possible different effects, as it is believed that the monomer is non-toxic, whereas the oligomers are thought to be highly noxious to cells; as for the fibrils, some authors defend that they represent an off-pathway in an attempt to avoid toxicity.

Finally, we also evaluated collagen IV levels in AD/TTR+/- IDIF-treated mice, a TTR tetrameric stabilizer. It is known that stabilization of TTR tetramer enhances its functions, such as Aβ cleavage (Ribeiro, Saraiva et al. 2012) and clearance (Alemi, Silva et al. 2017). We observed reduced collagen IV around brain vessels in treated mice as compared to non-treated, indicating that, in fact, TTR stabilization is also beneficial in this TTR-related effect.

Altogether, we propose that TTR exerts a neuroprotective function in AD by cleaving and binding Aβ, decreasing its accumulation in the brain, and avoiding its effect in the increase of collagen IV, which would then lead to a consequent increase of Aβ, similar to a "snow-ball effect", contributing to disease progression. Researchers believe that vascular alterations occur early in AD development and, therefore, TTR-based therapies are promising.

Conclusions and Perspectives

Given that: 1) TTR is able to modulate some CNS genes, including genes/proteins that are expressed at the BBB; 2) TTR facilitates Aβ transport across the BBB and 3) preliminary data indicated that TTR reversed the decrease in TJs proteins induced by Aβ (not shown), we engaged a study to determine if TTR is involved, in any way, in the brain vascular system.

As conclusion, we can say that TTR variants (WT, V30M and L55P), in physiological conditions, are 1) not capable to modulate the expression of TJs-related genes at the transcript and protein levels, and are 2) not involved in the regulation of angiogenesis in the brain, albeit 3) we confirmed its neuroprotective effect while affecting, even if indirectly, the levels of collagen type IV in brain vessels, thus facilitating Aβ elimination.

In fact, TTR had no effect on any of the TJs-related genes tested. However, our study was performed with a cell line and it does not mean that TTR does not modify the morphology of TJs, i.e. change the linkage between TJs. In the future, it would be interesting to address this point using isolated brain microvessels from mice with different TTR genetic backgrounds, allowing us to observe different intercellular tight junctions by TEM. Moreover, it would be important to evaluate the gene expression of other genes that presented a significant alteration in the PCR array.

In our study, it was also not possible to confirm the ability of TTR to regulate angiogenesis in hCMEC/D3 cells, as previously reported for HUVECs and for hRECs. Additional experiments should be performed before completely ruling out this hypothesis, such as the tube formation assay and in vivo Chick Chorioallantoic Membrane (CAM) assay as an in vivo tool to evaluate angiogenesis.

Our study suggests that TTR is involved in the regulation of collagen IV levels in AD. Although the literature refers that the thickening of the BM can lead to Aβ accumulation around brain vessels by preventing its passage to the blood, it is not clear what is the initial trigger. In this work, we tested the hypothesis of Aβ as a cause for the increase in collagen type IV levels. However, we could not determine if only a specific Aβ species is the responsible, or if even the soluble peptide can do this. Thus, homogenous preparations of soluble, oligomeric or fibrillar Aβ should be obtained and tested.

As a follow up of this project, it is important to test if increased collagen levels indeed affect BBB permeability to Aβ. To this end, permeability/transport studies should be performed, for instance by using hCMEC/D3 cells grown in transwell filters coated or not with collagen type IV and by measuring brain-to-blood Aβ transport. Although our AD transgenic model established in different TTR genetic backgrounds has already been characterized and shown that TTR reduction results in a more severe state of disease, detailed localization of Aβ accumulation in the brain was not performed. Thus, evaluation of Aβ localization and levels in brain vessels in these mice should be performed to correlate TTR/Aβ/collagen type IV levels. Moreover, and aiming at understanding which is cause and effect, evaluation of Aβ and collagen type IV levels in vessels should be performed in animals of different ages, before, at and after disease onset. Finally, isolated brain microvessels (after further optimization of the current protocol) might constitute an alternative model to measure the thickness of the basal membrane and to investigate the effects of Aβ and TTR, as well as other factors.

Alterations in the brain vasculature are thought to occur very early in AD and thus this work highlights the potential of TTR as neuroprotective protein and as a therapeutic target.

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